THE IMMUNOGENICITY

OF

INFLUENZA VIRUS VACCINES

Based on a study using laboratory mice.

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Statement

These studies were originally conceived by Professor Fazekas de St. Groth, and some of the work reported in Chapters II, IV, V, and VI followed the plan outlined by him. The large factorial experiments reported in Chapters III and V, and the initial investigations of the antibody response in mice described in Chapter IV, were jointly planned with Professor Fazekas de St. Groth.

All the experimental work was carried out entirely by the candidate.

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' But still it was more often those who had recovered who had pity for the dying and the sick, because they had learnt what it meant and were themselves by this time confident of immunity; for the disease never attacked the same man a second time, at least not with fatal results. And they were not only congratulated by everybody else, but themselves, in the excess of their joy at the moment, cherished also a fond fancy with regard to the rest of their lives that they would never be carried off by any other disease. '

Thucydides: Description of the Plague at Athens.

History of the Peloponnesian War. Book II. Ll.5

Translated by C. Foster Smith, Heinemann, London.

INTRODUCTION

Although the development of resistance to reinfection which follows recovery from, or exposure to, an epidemic disease, has been observed from the earliest times, the present concept of the nature of immunity is almost entirely the result of investigations carried out during the past century. In particular, a proper understanding of immunity did not become possible until the introduction of statistical techniques and correctly planned experiments allowed meaningful information to be derived from measurements of various biological phenomena which influence it.

The term immunity, as used in the study of diseases, no longer implies complete exemption from morbidity; it can be defined as : Some degree of acquired, specific, resistance to a pathogen (toxin, virus, bacterium, etc.). On the one hand, this definition* distinguishes immunity from non-specific temporary states of increased resistance which are induced by chemoprophylactics, or by interfering agents, etc. On the other hand, it also distinguishes it from those states of innate, or genetic resistance which have been described. e.g. Sabin (1952) showed that the inherited insusceptibility of inbred PRI mice to 17D yellow fever virus depended on a single pair of genes which depress the level of virus multiplication.

*Definitions of immunity which impart somewhat different meanings have been used by others, and it seems necessary to define clearly the meaning as used throughout this Thesis.

In keeping with this definition, a given procedure may be said to be "immunogenic" if it produces some degree of specific resistance to a pathogen. The word immunogenic may be applied, in the same sense, to the particular biological material employed to produce immunity to a specific disease, with the tacit assumption that details of procedure, such as route of injection, have been correctly chosen. It is therefore desirable to define an immunogen as a substance which, when introduced by an appropriate route into animals of a susceptible species, will elicit some demonstrable degree of specific resistance to subsequent challenge with a pathogen. This definition must be qualified in that the immunity so acquired is to be distinguished from states of immunity which can be induced passively by antisera, or by certain cell suspensions.

The terms immunogen and antigen are, of course, not synonymous although particular antigens (e.g. toxoids) may well act as immunogens. The outcome of infection does not entirely depend on the presence or absence of circulating antibodies, and indeed, the development of immunity following vaccination may well be unrelated to the simultaneous production of serum antibodies (Raffel, 1960). Assessment of the immunogenic potency of vaccines is dependent on the ability to observe that in vaccinated animals death, damage to cells, connective tissue, etc., or stimulation of such processes as oedema, have been prevented, or that the severity of symptoms have been modified in a measurable way. If the pathogen is an infectious agent,

multiplication may occur in challenged immunised animals, but they must show a reduction in symptoms or lesions when exposed to infection in a manner which in unimmunised animals of the same species would result in a clearly defined disease complex.

This Thesis examines the present position with regard to the study of the immunogenicity of Influenza virus vaccines, and reports original experimental work in this field. In Chapter I the factors which have been shown to be of importance in immunogenicity are discussed, and a brief historical outline of the approach to this problem in the case of typical living and killed vaccines is given. Chapter II reports some findings relative to the measurement of certain characteristics of the virus which are used to indicate immunogen content.

Chapter III reports a detailed study of those factors which influence the outcome of experimental influenza in the laboratory mouse, and Chapter IV is devoted to a similar study of the antibody response to vaccination. Chapter V reports the results of challenge tests used to investigate the immunogenicity in mice and Chapter VI will detail the results of experimental work designed to disclose the correlation between the outcome of challenge, and a number of vaccine characteristics which can be quantitated.

General discussions and statement of conclusions are given in Chapter VII and details of materials and methods are appended.

CHAPTER 1

IMMUNOGENICITY

INTRODUCTION

The study of immunogenicity has largely been inspired by the need to ensure, and the desire to improve, the efficacy of immunogens for the control of infectious diseases of man and animals. It is obvious that from these points of view the effectiveness of vaccines or toxoids will only be meaningful in the light of their ability to increase resistance to natural exposure to pathogens. Field trials would therefore appear to be the ideal means of studying immunogenicity and, indeed, rigidly controlled field trials, correctly planned and interpreted, remain the final arbiters of this effectiveness. Unfortunately, all too often the planning is carried out in retrospect, important factors may not have been considered, and the field trial may turn out to be an expensive means of obtaining misleading information.

Whilst proper field trials will always be required to reveal the basic type of information which they alone can give, they are unsuitable for experimental studies directed towards accurate measurement of immunogenicity. A major difficulty with field trials is that it is seldom possible to be sure of exposure to infection within the period when observations must be made; this practical consideration applies especially to all diseases of epidemic character. However, even in the most felicitous circumstances imaginable, where the disease is endemic, and if the members of the population at risk who have had previous exposure are known

and excluded from the trial, the test subjects will be exposed to different doses of pathogen, of unknown and perhaps differing virulence, at various times after immunization, and under different conditions of diet and environment. The results of such tests may only be regarded as qualitative; although the information they can reveal will be highly significant, and of vital importance to the understanding of particular aspects of immunity.

Most of the problems associated with "natural" exposure may be avoided by using challenge tests in which known doses of parasite are simultaneously used to infect the test species. The virulence of the parasites can be established by preliminary experiments and due precautions taken to ensure that each animal on test receives the same number of equally virulent challenge organisms, suspended in the same medium (See Pittman, 1941). The term virulence is used here, and later in this thesis, according to the definition of Miles (1955), and includes the sum of many attributes, e.g. invasiveness, necrotizing and cytocidal potency, which may vary with different strains of the same parasite, and which may be of varying importance with different members of the infected species. These components of virulence may decide the reaction of an individual host, be it inapparent infection (Lwoff, 1957) or a recognisable disease process, and the severity of the latter if it occurs.

So far as human diseases are concerned, challenge tests usually cannot be carried out in the host species in which we are interested. For economic reasons the same may be true of animal diseases, although here it is always possible to use a challenge test to establish the

fundamental effectiveness of an immunogen in the species for which we require a prophylactic. This method had its beginnings with Pasteur (1881) who demonstrated the efficacy of his attenuated anthrax vaccines in various farm animals. Although his results were only qualitative, they were valid. Pasteur also introduced the principle of controlling the virulence of the challenge bacteria.

In most studies of immunogenicity, the need to assay potency of immunogens has led to the use of laboratory animals which are not natural subjects of the diseases concerned. Routes of infection, known to differ from the customary routes of infection in the natural host, may be obligatory to produce morbid processes. In these cases, and even where the same route of infection produces disease in both species, the pathogeneses of the artificial and natural infections are seldom strictly comparable. Burnet (1960) has pointed out that many of the characteristics of their "immune response" will have been bred into species in the course of evolution. It is unlikely therefore that the mechanisms of natural resistance, and the responses to immunizing processes, will be identical in any two species.

Nevertheless, a challenge test in animals remains the best means available for the direct study of immunogenicity; the equating of serum antibody titres with immunity levels is unjustified without supporting field evidence (Fazekas de St. Groth, 1963). If care is taken in the selection of the laboratory animal, and if the test system has been shown to correspond at least qualitatively with the disease process and immunity reactions of the natural host, then it is reasonable to assume

that estimates of immunogenic potency by means of experimental challenge tests can be validly applied to the natural host species.

FACTORS INFLUENCING IMMUNOGENICITY TESTS

For the proper performance of challenge tests the control of a number of factors is of fundamental importance. These may be inherent characteristics of the host and parasite, or they may be external conditions, such as diet or environmental temperature which influence the host's response or the virulence of the parasite.

With regard to host characteristics, it is a commonplace observation that if properly graded challenge doses of pathogen are administered to a group of unimmunized susceptible animals, the individuals will be found to vary in their reactions to the infectious process. Whilst these variations will be minimal at the extremes of challenge dose, there is a range of doses over which it is usually possible to distinguish discrete levels of morbidity in animals which have been infected with the same amount of pathogen. In certain types of fulminating infections, such as follow the intracerebral inoculation of French neurotropic yellow fever, and some other arboviruses, in mice, it is often impossible to make observations beyond death or survival in groups of animals challenged with the most closely spaced dilutions of pathogen. However, even in these cases, it is not unusual to find extremely susceptible and insusceptible animals in the population subject to challenge.

The dose required to produce death in individual animals of

random breeding may vary by several orders of magnitude. This variation was an insuperable obstacle to early workers on immunogenicity because, unfortunately, they chose the concept of the minimum lethal or effective dose as the unit for quantitative studies. Ehrlich (1897) devised this unit to measure toxicity, and considered himself fortunate that he was able to achieve a reproducible estimate of the minimum lethal dose (M.L.D.) of diphtheria toxin in guinea pigs. He was aware that the reason for his failure to obtain a reproducible estimate of the M.L.D. in other systems such as tetanus toxin, snake venom, ricin, in various hosts, was due to the much greater variation in the host response in these systems (Croonian Lecture, 1900). As Miles (1954) has pointed out, the results that Ehrlich obtained using this unit were valid for the study of the chemical combination of diphtheria toxin and antitoxin, and permitted a number of important discoveries. However, his success appears to have encouraged others to apply the M.L.D. concept to other systems, with unfortunate results due to the inaccuracy which accompanies measurement of this unit where there is variation in the host response.

From a biometric point of view the problems associated with host variation in challenge tests were not solved until Trevan (1927) described the results of such tests in terms of dose-response curves, and then proceeded to develop the $L.D._{50}$ as a unit. This and similar units, generalized as the effective dose or $E.D._{50}$ have allowed the development of assay systems whose accuracy may be estimated; calculations of potency based on such systems can be tested for validity. Nevertheless, the accuracy of all bioassays is inversely proportional to

the amount of variation in the host reaction, since this decides the slope of the dose response curve. For this reason it is advisable to use inbred lines of animals where these have been found to show less variation than random bred lines in their response to either the immunogen or the pathogen. Pittman (1941) found that it was essential to use closely inbred mice in challenge tests with meningococci. Gowen (1950) developed a series of inbred mouse lines which have graded but fixed susceptibilities to mouse typhoid; and this raises the possibility of breeding laboratory animals with responses to infection which closely resemble the corresponding disease in humans or other animals. Again, since strains of mice have been bred which differ in their capacity for antibody production to different antigens (Fink and Quinn, 1953), it may be possible to incorporate the most appropriate antibody response in the breeding of the test animals. Russell (1955) briefly reviewed the reasons for using carefully inbred lines in research involving animals. However, it should be mentioned that inbred lines of animals are not always superior to random bred animals. With currently available lines, there may be reasons for preferring hybrid or random bred animals in particular bioassays. McLaren and Michie (1954) have shown that the inbred C57Bl strain were more variable in their response to a narcotic drug than either F_1 hybrids from C57Bl♀ x C3H ♂ crosses or a random bred albino strain.

Variation in the virulence of the challenging organism can be minimized by preparing a sufficiently large "pool" of a pre-tested strain, and storing this in a manner which will maintain full infectivity and virulence. The techniques of preparation of the

challenge material, the cleanliness and accuracy of the apparatus used are of such obvious importance to both virulence and dose received by the test animals that they need no more than a mention. The medium used for suspension of the dose may also be a critical factor in the stability of virulence and infectivity, as was shown in the case of typhus rickettsiae (Bovarnick et al. 1950).

The choice of the dose level to be used in challenging immunized animals is also of critical importance if variation in the response is to be minimized. Batson (1950) has convincingly demonstrated the wide variation in dose which will actually be received by the test animals unless the challenge level is large enough to overcome the effect of the Poisson distribution of organisms in the test material. The serial dilution which is customarily used in adjusting the challenge to the right level will also result in actual final concentrations which have a Poisson distribution. This will not have a significant effect provided that the dose level is reasonably large - 1000 $E.D._{50}$ units is desirable but 100 is usually sufficient for the accuracy required in most systems.

Other factors which have been found to influence immunogenicity tests include age, sex, and weight of the test animals, their nutritional status and physical environment, and the interactions of these factors. A general discussion of their importance will be given in later chapters in conjunction with their particular association with influenza virus studies.

PLANNING OF IMMUNOGENICITY TESTS

With regard to the correct planning of immunogenicity assays, Jerne and Wood (1949), advised by Finney, have discussed the assumptions and implications behind biological assays and, drawing freely on the published reports of other workers, have proposed rules of conduct for the design and performance of such experiments. In any planned study of immunogenicity it will be necessary to comply with these rules if valid conclusions are to be drawn from the results. The most important requirements are :-

(1) Adequate preliminary biological research into the particular system to be studied:

(2) Elimination or control of factors, other than the dose of immunogen, which may influence the response being measured:

(3) Selection of the correct doses of immunogen and challenge material and accurate measurement of these and also of the response:

(4) Selection of the most appropriate mathematical processes, to provide maximum utilization of the available data:

(5) The functions relating dose and response measurements must have the same form for each of the substances being compared.

Other matters may be considered in regard to increasing the accuracy of estimates of immunogenic potency. Fisher (1935) directed attention to the necessity of designing bioassays in such a way that the interactions of the factors which affect the response can be determined and separated from the main effects, thereby increasing the

accuracy of interpretations. He also highlighted the necessity of proper randomization - where appropriate - of the test subjects. Measurements of the responses must always be made without bias, conscious or unconscious, on the part of the operator, and it is advisable to make them as objective as possible. In this respect experiments should be planned so that the operator is not aware of the identity of the doses he inoculates nor of the dose levels which have been received by the animals he examines. Mathematical transformations are usually employed to simplify the dose-effect relationship, and permit improved statistical analysis of the experimental data, thereby giving more precision to estimates, and enabling the determination of limits to these estimates.

HISTORICAL DEVELOPMENT

It will not be possible to review the histories of immunogenicity studies in all vaccines , but the work of the earliest experimenters in this field will be examined, and subsequent developments in the case of a typical "living" and "killed" vaccines will be described.

Living Attenuated Vaccines

The study of immunogenicity began with the first planned development of attenuated living vaccines. Pasteur (1881) produced B. anthracis strains of altered virulence for small animals by means of continued daily sub-culture on artificial media at 42-43 C. To achieve a state of immunity in farm animals to subsequent challenge

with fully virulent organisms, he found it necessary to use as a primary vaccine, a suspension of a strain which was still capable of producing a high mortality in mice and young guinea pigs, but which would not kill mature guinea pigs. A second vaccination was required 12 days later, with a strain which killed adult guinea pigs, but had only a limited mortality in rabbits. A strain which was so attenuated that it was no longer lethal for mice, was not immunogenic.

Haffkine (1892) introduced challenge tests in inoculated guinea pigs to prove the immunizing power of his attenuated cholera vaccines intended for human use, and Tamamcheff (1892) employed this method to compare the effectiveness of attenuated and killed V. cholerae vaccines. Although Haffkine (1893) felt that humans would react to his vaccines as rodents had done, and planned his Indian field trial on that basis, he stated nevertheless that the final test of immunogenicity in humans would have to be carried out in humans, under natural conditions of exposure.

Equating of the infectivity of the preparation with immunogenicity was the background to the development of a "standardization test" for smallpox vaccine by Calmette and Guerin in 1901. This qualitative test gauged the amount of vesiculation produced on the shaven skin of a rabbit by 1 ml of vaccine, and assumed that an arbitrary degree of vesiculation corresponded with a satisfactory level of "virulent elements". Calmette and Guerin reasoned that smallpox vaccines should produce 100% "takes" in children to be satisfactory,

and that, to do so, they must contain sufficient "virulent elements".

Potency testing of smallpox vaccines became quantitative when Guerin (1905) introduced the idea of testing serial dilutions, and was thereby able to make crude estimates of the relative potencies of various preparations. With slight modifications, his test survives to the present day as one of the recognized means of assessing potency of smallpox vaccines, since the shaven skin of the rabbit is extremely sensitive to vaccinia virus, and no more than a crude estimate of virus content has been required for standardization purposes. Even if the content of "virulent elements" is still to be regarded as the sole criterion for immunogenicity of living vaccines, then in the case of smallpox there is good reason to abandon this technique, because the simpler and more accurate infectivity tests in tissue culture, or on the chorioallantoic membranes of embryonated eggs, may be substituted instead.

With regard to the relationship between infectivity and immunogenicity, it is obvious that sufficient "living" organisms must be present to overcome the threshold of natural resistance to infection. After this has been achieved, the level of immunity ultimately reached will be a function of , (a) the increase in numbers of the invader with concomitant production of much greater amounts of potential immunogen, (b) the extent to which the inherent characteristics of both the invader and the individual host will permit the immunogen to reach those sites which will be productive of immunity, (c) the qualities of the

immunogen and (d) the resistance of the host to the toxic effects of the invader, and its metabolic products. The experiments of Cockburn et al. (1957), who were able to correlate infectivity on the chorioallantoic membrane of eggs with percentage of takes in a small group of male humans, have led to a blanket "potency" recommendation of a minimum infectivity of 10^8 P.F.U. per ml. for all smallpox vaccines. Too much confidence should not be placed on this specification because it ignores variation between vaccinia strains in elementary body - P.F.U. ratios (Joklik, 1962), and in their infectivities for different animals (Fenner, 1958). It is most likely that different strains of this virus will differ in their infectivities in humans, and also that different races of men will not all have equal sensitivity to infection.

At the present time the only test for the development of immunity after smallpox vaccination is by means of revaccination, and this has been used by Cross et al. (1958) to show that the level of infectivity on the chorioallantoic membrane of eggs did not influence the immunogenicity of the vaccine provided that sufficient virus was present to bring about the formation of a typical pock in human subjects. This is in keeping with the observation of Gatti (1764) that even if variolation produced only one pustule, it successfully protected against smallpox which was endemic in Europe at that time. What relationship, if any, exists between resistance to revaccination and immunity to smallpox is not known. Soloviev and Mastyukowa (1959) differentiated the immunogenicity of several vaccinia

strains for rabbits challenged with neurovaccinia by the intracerebral route. The relative levels of immunity produced in rabbits appeared to correlate with certain aspects of the virulence of the particular strains in humans. Dixon (1962) has proposed that there should be more field and laboratory tests of smallpox vaccines on a larger scale than heretofore, so that the immunogenicity of vaccine strains can be properly determined. In the present state of ignorance, the customary habit of revaccinating without exception all who may be exposed to smallpox, is a very wise procedure.

The assumption that there is a direct correlation between infectivity, measured in some arbitrarily chosen bioassay, and immunogenicity in human or animal hosts is still the basis for the standardization of most attenuated vaccines. An exception is Rabies vaccine for which a challenge test is prescribed (W.H.O. Monograph, No. 23), and it may be significant that this test was developed by Habel (1940) following a number of critical investigations of the effectiveness of rabies vaccines, (Webster,1939).

Killed Vaccines

The history of the study of immunogenicity of killed vaccines also had its beginnings with Haffkine's early attempts to prepare cholera vaccines, and Tamamcheff's (1892) use of a challenge test in guinea pigs to compare the effectiveness of living and killed vaccines. The problems attached to this study, and the pitfalls awaiting the unwary experimenter in this field are well illustrated

in the case of typhoid vaccines. A brief survey of this history is most illuminating. The success of Haffkine's anticholera field trial in India, reported in 1895, inspired Wright and Semple (1897) to prepare a heat killed vaccine from S. typhi which was adjusted to contain a standard number of organisms, but was only considered "potent" if the bacterial suspension from which it was made had an arbitrarily chosen minimum lethal dose in guinea pigs. In 1900, Wright and Leishmann used maximal virulence for guinea pigs as a sine qua non for seed strains to be used in typhoid vaccine preparation, and potency testing also required the demonstration of definite levels of toxicity of the killed VACCINE in those animals as well as a standard content of organisms. The concept that virulence of the vaccine seed strain determines the immunogenicity of a killed vaccine was taken by analogy from Pasteur's results with attenuated cultures, and is still the basis for some theorising on the effectiveness of killed vaccines (Salk, 1956). Wright and co-workers also used the agglutination test (Charrin and Roger, 1889) (Gruber and Durham,1896) to follow the development of antibodies in human volunteers. The type of agglutination which would have been assayed was for the H antigen, although at that time the subtleties of antigenicity had not been discovered. During the early years of this century challenge tests in guinea pigs were used to indicate immunity. These culminated in the method of Vincent (1910) in which 3 to 4 subcutaneous injections were followed after 15 days by intraperitoneal challenge with a virulent culture, plus a subcutaneous injection of 2 - 4 mls. of 10% NaCl containing 0.1 ml of

Aniline. Metchnikoff and Besredka (1911) in reporting their experimental work in chimpanzees spoke of all such tests with derision : " All that is common to human typhoid fever and intraperitoneal typhoid in guinea pigs is the name of the microbe." Vincent (1911) taking part in the discussion following the presentation of their paper, maintained that results obtained in humans must take precedence over ANY animal experiments. He pointed out that his empirical use of injections of hypertonic saline in the test guinea pigs produced a disease syndrome which closely resembled human typhoid. Subsequent experimental work reverted to the use of simple experimental challenge with living organisms - in guinea pigs, and later in mice (Grinnell, 1932).

According to the British Army Council Anti-typhoid Committee (Report 1912) the results of their investigations showed that the virulence of the strain of bacterium used for vaccine preparation was unimportant. Their criteria for the effectiveness of typhoid vaccines were the content of organisms and the ability of these vaccines to elicit the formation of agglutinating (H) antibodies following parenteral inoculation.

This finding was challenged by Felix (1924) mainly on the grounds that H agglutinins had been found in the sera of human typhoid cases which ended fatally, but the somatic O antigen agglutinins were always absent. Arkwright (1927) found that the presence of the H antigen in vaccines was of no significance in increasing resistance to simple intraperitoneal challenge in guinea pigs, and a similar finding

for the O antigen was claimed by Grimmell (1932) using mice. The isolation of the O antigen in relatively pure form and the demonstration of its immunogenicity in mice by Felton and Wakeman (1937), as judged by intraperitoneal challenge with virulent S. typhi suspended in mucin (Rake,1935); restored the O antigen to favour as the important immunogen. It was joined by the Vi antigen of Felix and Pitt (1934) which is only found in virulent strains, and was credited with immunogenic function largely on the basis of its ability to elicit serum antibody with high neutralizing activity in passive protection tests, also carried out in the mouse peritoneum.

On the basis of their work, a new type of vaccine, killed with alcohol, was used by the British Armed forces after 1943 (Felix, 1951), although disquietingly severe outbreaks of typhoid occurred in 1950 in a Royal Air Force unit at the Suez Canal (Marmion et al. 1953). The Yugoslav Typhoid Commission in conjunction with the World Health Organization, and the U.S.Public Health Service, set up a well planned field trial which operated from 1954 to 1960 to investigate typhoid vaccines in an endemic area. The results (Edsall et al. 1959, Standfast, 1960, and the Yugoslav Typhoid Commission, 1962) showed that the usual laboratory tests in mice, both active intraperitoneal challenge, and passive neutralization, as well as an intracerebral challenge test, failed to distinguish a heat killed vaccine, which was highly immunogenic in humans, from an ineffective alcohol vaccine prepared from the same bacterial suspension. The only correlation was shown by a test for anti-H serum antibody production in rabbits. From this history it

is obvious that any attempts at direct translation of the results obtained in studies of disease processes in laboratory animals, which do not strictly parallel the corresponding syndromes in the natural host, may well lead to misleading deductions. This is not to say that experiments designed to measure immunogenicity of typhoid vaccines in mice and guinea pigs have so far been unproductive in the study of immunity. Very many of the fundamental aspects of bioassay have had their practical development in these systems, e.g. Batson (1950), and the work of Felix and his colleagues has greatly advanced our understanding of the nature of antigens. Moreover, much is still to be gleaned from studies carried out in such systems. Successful interpretation of experimental findings will only be possible if the hypotheses on which the experiments have been planned have factual bases. Where these hypotheses also involve assumptions of identity of reaction between different biological species, the outcome of the experiments must, of necessity, be treated with reserve unless definite proof of that identity of reaction is available.

Unfortunately, in the case of typhoid vaccines, the evidence for the importance of the various antigens in human immunity was always tenuous, and the tests used since the first world war were really directed to measuring the "antigenicity" of various preparations.

IMMUNITY TO INFLUENZA VIRUS INFECTION

Active immunity to the effects of influenza virus infection was first demonstrated by Shope (1932) in relation to Swine influenza virus in pigs. Animals which had received live virus, either by the

intranasal or the intramuscular route, did not suffer the severe, and sometimes fatal, illness which follows combined intranasal challenge with the virus and Haemophilus influenzae suis. Wilson Smith, Andrewes, and Laidlaw (1933, 1935), and later Francis and Magill (1935), published reports of similar observations using viruses of human origin in ferrets and mice. The Commission on Influenza (1943) showed that immunity to reinfection, with the same type and strain of virus, might follow exposure to infection in human subjects, regardless of the occurrence or otherwise of clinical disease. This immunity was found to wane with time (Francis et al. 1944), and was accompanied by the presence of neutralizing antibody in the circulation (Henle, et al. 1946).

Stokes et al. (1937a) found that subcutaneous inoculation of live "human" virus in a group of human subjects, shortly prior to natural exposure, led to a significant reduction in febrile cases, as compared with similar groups inoculated with "swine" virus, or left untreated. This immunity and the presence of neutralizing antibody in serum were found to be associated, but absolute correlation was lacking; a similar finding had previously been made by Wilson Smith et al. (1935) working with human virus in ferrets, and by Shope (1936) with swine influenza in pigs. Somewhat better correlation was found in challenge tests with human volunteers in later experiments in which the infectious dose was properly controlled (Burnet and Foley, 1940; Henle et al.1943.) In 1939, Burnet et al. showed that human nasal washings contained some factor which reduced the infectivity of a variety of viruses, including

influenza. Francis (1940), confirming this discovery, found an association between the ability of human nasal washings to inactivate influenza virus, and the neutralizing capacity of sera from the same individual subjects. The "virus inactivating substance" was shown to have some biochemical properties akin to the antibody fraction of serum, although it was peculiarly sensitive to mildly alkaline pH. (Francis ,1941a, 1941b). It was also greatly increased in washings from convalescent influenza patients. Francis et al. (1943), finding that it was also increased following parenteral inoculation of either living or killed vaccines, and that the increase was roughly correlated with a similar increase in serum antibody, concluded : "high levels of circulating antibodies may serve to prevent disease by virtue of the enhanced amount of antibody which becomes available in the nasal secretions".

Using immunized mice, Fazekas de St. Groth and Donnelley (1950a) found a strong positive correlation between resistance to specific challenge, and the anti-haemagglutinin titres of bronchial washings. The levels of immunity, and of bronchial anti-haemagglutinin, were closely correlated with the amount of vaccine injected by subcutaneous, intraperitoneal, or intranasal routes, and were specific for the type of virus in the vaccine. Subsequently, the same authors (1950b) and also in conjunction with Graham (1951) showed that the intranasal instillation of some irritants in immunized mice produced a considerable increase in the level of anti-haemagglutinin in the bronchial washings, and a proportional increase in the level of immunity to

challenge. These irritants were inactive in unimmunized mice, and were only effective at levels which caused mild inflammation, and a resultant increase in tissue, and sometimes capillary permeability. Fazekas de St. Groth (1951) went on to prove that the same effect could be produced in immunized mice if dicoumarin was added to their diet. From this evidence it appears that mediation of the results of challenge in actively immunized mice is effected by means of the accumulation of specific antibodies at the site of infection, although these antibodies have probably arisen at some other location (Burnet and Clark,1942; Inglot and Davenport,1962), and may be at a much higher level in the general circulation (Fazekas de St. Groth and Donnelley, 1950 b).

Although it has not been possible to study in such detail the mechanism of active immunity in human influenza, the evidence of Francis and his coworkers, cited above, is usually taken to indicate that the situation is the same as that shown for mice. Presumably, antibody acts by combining with virus in a manner which prevents or limits infection of susceptible cells and subsequent spread to fresh sites in the respiratory tract. Burnet (1960) points out that little experimental work has been done to study the possible influence of antibody on virus already within a cell, and this remains an open question.

For the same reason it is not possible to state what part cellular and other defense mechanisms play in resistance to influenza.

Consideration of their significance in immunity to influenza is therefore largely speculative, although a reawakening of interest in the pathogenesis of influenza, and the role of leukocytes in this disease, has resulted from the development of fluorescent antibody techniques (Hers et al. 1962; Inglot and Davenport, 1962).

Since the work of Stokes et al. (1937a), referred to above, a great many reports have appeared on the efficacy of influenza vaccines in preventing or modifying the natural disease in man. The subject has been well reviewed from time to time (e.g. Francis, 1953; Davenport, 1961) and a detailed discussion here is unnecessary. From a study of those clinical trials which have been properly planned and executed, it has been concluded: that immunity may follow the parenteral inoculation of living or appropriately inactivated vaccines, provided that certain requirements relating to potency and specificity are met (Francis, 1953);and that immunity, so produced, tends to wane with time (Francis, 1959). It seems likely that multiple injections of vaccine may increase the level of specific immunity, and also broaden it to include immunity to related strains of virus (Davenport, 1961).

Development of immunity following intranasal spraying of living attenuated influenza virus vaccines has been studied in humans by groups of workers in Australia (Burnet, 1943), America (Francis,1950), England (Isaacs and Roden, 1956), and Russia (Zhdanov and Soloviev,1962.) The published results are very contradictory, but apparently it is difficult to establish infection with attenuated strains where there

are detectable levels of pre-existing antibody (Meiklejohn, 1961).

IMMUNOGENICITY OF INFLUENZA VIRUS VACCINES

As in the case of killed anti-typhoid vaccines, two main lines of approach have been adopted in the study of the immunogenicity of influenza virus vaccines. Stokes et al. (1937 b) used an infectivity test in mice to check the potency of the live vaccine which they used for parenteral injection in humans. From the discussion in this paper it appears that their intention was to maximize the antigenic content of the vaccine so that a high level of neturalizing antibody would be developed in the volunteers. At that time it was not known if immunogenicity would be lost if the virus was inactivated, and an infectivity test was also regarded as a means of checking this aspect.

With the adoption of inactivated vaccines in the interests of safety, titration of haemagglutinin became the means of measuring antigen, and has remained one of the two currently used methods. Equating of haemagglutinin antigenicity with immunogenicity has been justified for influenza vaccines because of the general association between serum antibody levels and immunity as mentioned above, and which is illustrated in the studies of Francis et al. (1945) and Salk et al. (1945). Unfortunately, there is some evidence that haemagglutinin and immunogenicity are not strictly correlated (Pearson,1944; Henle and Henle,1947), but from a practical basis the haemagglutinin content is an approximation of the level of the protective antigen in influenza vaccines.

Several different biological systems have been used to assess the antigenicity of influenza vaccines, and the potency of commercial products is usually controlled by neutralization tests in mice or eggs. The Biologics Control Laboratory of the U.S.A. requires producers of vaccines in that country to use an intranasal neutralization test in mice, based on the work of Eddy (1947) which has rightly been criticised by Henle and Henle (1948) because it employs the titration of pools of mouse sera. Where arithmetic mean values are obtained, as must apply in this case, the results are often misleading because an exceptionally good response in one or two animals may create a false picture of high potency. The alternative test proposed by the Henles, which is based on protection of mice challenged intravenously, suffers from a marked variation in "toxicity" of influenza viruses by this route. Blaskovic and Salk (1947) developed a test using chick embryos with a α-procedure neutralization (Marrack, 1938), which is therefore lacking in precision. All these tests measure antigenicity of influenza virus in mice, and although this is a valuable study in itself, its direct relevance to the determination of effectiveness of vaccines for human use has not been demonstrated.

Immunogenicity of influenza virus vaccines in mice has also been investigated by means of challenge tests, designed to assess the potency of vaccines which were intended for human prophylaxis. By far the greatest development of this study took place in the U.S.A.

in the period before the Eddy test came into being. The outcome of intranasal challenge in mice has been assessed by mortality (Francis 1939), extent of lung damage (Bodily et al. 1943), a combination of mortality and extent of lung consolidation in survivors (Horsfall 1939) and a refinement of the latter which also incorporates survival time (Stone 1948). Lauffer and Miller (1944) showed by statistical analysis that the combination end-point sometimes referred to as the $M.S._{50}$ or $L.S._{50}$ was superior for PR8 virus to either simple LD_{50} or a similar unit for extent of lesions. A number of workers, Francis (1945), Stanley (1945), Salk et al. (1940) used the LD_{50} end point to measure the response of mice to graded doses of vaccine in multiple schedules of inoculation; mortality was recorded ten days after challenge. Bodily et al.(1943) used graded single doses of vaccines and appear to have used the lesion endpoint; and Friedewald (1944) adopted the composite end point of Horsfall in testing the immunogenicity of adjuvant influenza vaccines. With the latter as an exception, all workers used the intraperitoneal route for injection of the vaccines.

Fazekas de St. Groth and coworkers (1950,1951) in their investigation of the immune mechanism in mice, referred to above, used the refinement of the composite method described by Stone (1948). The arithmetic means of the estimated "percentage" damage in groups of mice at each dose level were transformed to probits. By this means they were able to calculate and compare levels of "protection" which resulted from different treatments in the mice. Since their papers

appeared in 1951 the systematic study of the immunogenicity of influenza vaccines in mice has not been pursued, although some workers (Boudreault and Pavilanis.,1961) used active challenge tests in these animals in work on individual vaccine preparations.

Examination of earlier work on immunogenicity of influenza virus vaccines showed that there were many aspects of this subject which had not yet been thoroughly studied. The work reported in this Thesis was planned to re-examine and expand existing information, using modern immunological techniques.

CHAPTER 2

ASSAY OF VIRUS IN INFLUENZA VACCINES

In the process of vaccine preparation it is customary to regulate the content of immunogen by estimation of the concentration of organisms. Although assessment of the number of bacteria or viruses which are present does not necessarily provide a measure of immunogenic potency, it is a pre-requisite for valid comparisons of the immunogenicity of different strains of organism, or different methods of preparation of vaccines. In the case of influenza virus vaccines, since infectivity titrations can only be used in special circumstances, estimation of the virus content is, in practice, based on assay of the haemagglutinin, although the relationship between haemagglutinin titre and particle counts made by electron microscopy has been shown to depend on the strain of virus and the individual erythrocyte suspension (Isaacs, 1957). However, since the proportion of filamentous to spherical forms varies with different strains, and even passage lines of virus, and the relative content of immunogen in the two forms is unknown, there is no valid reason for preferring particle counts to haemagglutinin titres as approximate measures of potential immunogen content.

Assay of Haemagglutinin

A number of methods have been adopted for the assay of haemagglutinin content, and these use either a "photometric" or a "pattern" type test.

Photometric titration methods measure the ability of virus preparations to increase the sedimentation rate of chicken

erythrocyte suspensions as a result of agglutination. The reactants, suitably diluted, are mixed in a standard diameter tube, and the cells are allowed to sediment through a fluid column of standard height during a period of 75 or more minutes. At the end of this settling time, the light scattering effect of the cells remaining in the middle portion of the tube is measured in a photo-electric densitometer, thus providing an objective estimate of the haemagglutinin content.

In the case of pattern tests, serial dilutions of the virus containing material are made in small volumes in tubes or the depressions in plastic trays. A standard quantity of chicken erythrocytes is added to each dilution, and agglutination of the cells results in the formation of characteristic patterns on the bottom of the tubes or plastic containers. These patterns are usually "read" after the lapse of 30 to 45 minutes, and titration endpoints are estimated on the basis of interpretation of the patterns of partial agglutination (Hirst, 1942).

Both types of test have been found to give results which are seriously influenced by factors other than virus concentration. Miller and Stanley (1944) have investigated the standard photo-metric titration (Hirst and Pickels, 1942) which has been designated the CCA (chicken cell agglutination) test. They found that age, concentration, and source of erythrocytes, ambient temperature, and to a lesser extent pH of the diluent, could have a

fundamental bearing on the titration result. The effects were so great that it was necessary to establish a standard vaccine preparation to be used as a control with all assays. Pattern tests have also been shown to be sensitive to changes in the ambient temperature (Salk, 1944), and titres vary with erythrocytes from different fowls (Anderson et al. 1946). It is common experience that "in vitro" age of cells may influence pattern haemagglutinin titres, and most workers in this field do not use cell suspensions beyond 48 hours after they have been taken from fowls. Reading of pattern endpoints is subjective, and may vary with different workers. A combination of these factors is probably responsible for the 5-fold difference between the titration results obtained by Finter at the University of Pennsylvania and Isaacs at Mill Hill in England, using the same virus preparations (Isaacs, 1957).

Assay of Enzyme Activity

As a result of the development of the Warren (1959) and Aminoff (1959) tests for free sialic acid, another method is available for estimating the concentration of myxovirus suspensions. This is based on the ability of these viruses to enzymatically release sialic acid from a variety of substrates as divergent as sialyl - lactose and urinary mucoprotein. Jacobs and Walop (1961) and Warburton (1961) reported that enzyme activity as measured by chromophore production in the Warren test, is proportional to virus concentration. Using sialyl-lactose, the method of estimating virus

concentration by means of enzyme units was found to be accurate and reproducible, provided that the virus preparation had been purified by at least one cycle of adsorption on and elution from chicken erythrocytes. However, enzyme activity is extremely strain dependent (Howe et al. 1960), and enzyme methods for assaying virus content are only of use in comparing preparations of the same strain.

Apart from this limitation, enzyme methods have the advantage of employing stable substrates which may be prepared in a relatively pure form, whereas haemagglutination tests are extremely susceptible to the variability of the erythrocyte suspensions.

The work to be described in the following chapters required the use of an *in vitro* test for approximation of immunogen concentration. It seemed advisable therefore to compare the effectiveness of the available methods, and a number of experiments were carried out to examine their precision, reproducibility, and correlation with virus content.

Methods and Materials

Pattern haemagglutination titrations were performed according to the method of Fazekas de St. Groth and Graham (1955). Volumes of 0.25 ml. of physiological saline were delivered into all depressions of transparent plastic trays, except for the first

column which contained 0.6 ml. of the primary dilutions. Serial twofold dilutions were then made by the aid of a 0.25 ml. spiral loop (Takátsy, 1950). A standard drop containing 0.025 ml. of 5% fowl erythrocytes was added from a calibrated dropping pipette; the trays were gently shaken and left to stand at room temperature for 30 minutes. The cell patterns were then read, and end-points estimated according to an arbitrarily selected degree of partial haemagglutination.

Photometric titrations were carried out using the Miller and Stanley (1944) modification of the Hirst and Pickels (1942) CCA test, and a PR8 virus preparation, supplied by the Division of Biologics Standards, N.I.H., Maryland, U.S.A., was used as the "standard" virus. Serial twofold dilutions of virus were made in 1.0 ml. volumes in 7 x 0.8 cm. tubes, carefully selected for lack of imperfections, using 0.85% sodium chloride buffered with 0.01M phosphate as the diluent. One ml. of a 1.5% fowl erythrocyte suspension was added to each tube with thorough mixing, and after standing for 75 minutes the optical density of the cell column in the middle area of the tube was measured. Interpolation of the results to a point which corresponded to a figure obtained with the standard virus preparation, titrated at the same time, provided the CCA titre of the test virus suspension.

For the enzyme test, sialyl-lactose was prepared according to the method of French and Ada (1959), except that the final material

was dissolved in 30 volumes of distilled water, and brought to pH 7.2 by the careful addition of 0.1M potassium hydroxide. A 2% solution of the potassium sialyl-lactate was stored in the frozen state at -20°C, and 0.14 % solutions in calcium saline (see Appendix 1) were prepared as required from this material.

Virus suspensions were prepared by the inoculation of approximately 10^3 ID_{50} of virus into the allantoic cavity of 11-day old chick embryos, and incubation for 2 days except in the case of B/Lee which was grown for 3 days. The allantoic fluid was then harvested and concentrated 10-fold by one cycle of adsorption to and elution from 2% human erythrocytes. Differential centrifugation was used to achieve 2 further purifications, and the virus was finally resuspended in calcium magnesium saline (see Appendix 1) to which 0.08% sodium azide was added as a preservative.

Enzyme tests were carried out in the following way: 0.25 ml. aliquots of 0.14% sialyl-lactose in calcium saline were delivered into 5 x $\frac{1}{2}$" test tubes by means of calibrated dropping pipettes, and the tubes were transferred to a water bath adjusted to 37 ± 0.2°C. Then 0.25 ml. of virus, suitably diluted in calcium saline, and pre-warmed, was added at intervals of at least 10 seconds, with shaking to achieve rapid and complete mixing with the substrate. Ten minutes after the addition of virus, 0.25 ml. of the periodate reagent of Aminoff (1961) was mixed in rapidly, using the same time interval between tubes, and free N-acetylneuraminic acid was subsequently estimated according to the method developed by that

author. The alternative method of Warren (1960) may be used, but was not preferred because it employs cyclohexanone which can be toxic for humans.

Duplicate control tubes with sialyl-lactose and extra diluent were incorporated in the test to allow for the estimation of the background colour. Both the Aminoff and Warren methods show appreciable chromophore in blank determinations, apparently due to the slow release of N-acetylneuraminic acid from sialyl-lactose during the preliminary periodate oxidation step. Since a considerable excess of unchanged substrate was always present in each tube, there was no problem of variation in the background due to enzyme action. Controls with the highest concentration of virus and extra diluent were included in all tests, but were universally without colour formation. The range of virus concentrations was always selected to provide readings between .200 and .800 in the Beckman Spectrophotometer, and the means of duplicate readings, from which the blank readings had been subtracted, were multiplied by 100 to give virus enzyme units. The units were, of course, distinctive to each virus strain.

COMPARISON OF PRECISION AND REPRODUCIBILITY

In the paper by Miller and Stanley (1944), detailed results are given of 30 individual CCA tests. The figures supplied are the mean end-points of duplicate titrations of two vaccines, and it was therefore not necessary to carry out a further large series of tests to obtain an estimate of the standard error of this method. After

TABLE II - 1

REPRODUCIBILITY OF PATTERN TEST

Reciprocal titres of replicate titrations of a B/Lee virus suspension. Tests performed as duplicate titrations with 3 primary concentrations of virus.

PRIMARY DILUTION	RESULTS OF INDIVIDUAL TITRATIONS	MEAN OF RESULTS OF DUPLICATE TITRATIONS
Undiluted	109.5 117.5	113.5
	117.5 117.5	117.5
	125.9 125.9	125.9
	117.5 117.5	117.5
1/4	125.9 125.9	125.9
	125.9 125.9	125.9
	125.9 117.5	121.6
	125.9 117.5	121.6
1/16	125.9 125.9	125.9
	125.9 125.9	125.9
	117.5 125.9	121.6
	134.7 125.9	130.3

adjustment for the different concentrations of virus which were used, the standard error for mean duplicate titrations was found to be 4.12%.

Fazekas de St. Groth and Graham (1955) state that the error of a single titration by their pattern method is about 8%. No detailed results are given, however, and this test was therefore investigated to obtain an estimate of the error which might then be used for comparison with the other methods. Twelve duplicate titrations were performed using a B/Lee virus preparation. Primary dilutions of 1/4 and 1/16 were used for each of 4 of the duplicate titrations, and the remaining 4 were carried out with undiluted material. Table II-1 shows the results expressed as reciprocals of individual titres and also the mean reciprocal titres of the duplicate tests. The titres obtained with the 1/4 and 1/16 dilutions were adjusted to be comparable with the results obtained with the undiluted material. The standard error of the mean duplicate titrations was found to be 3.89%, which is comparable with the figure for the error of the CCA photometric test.

The enzyme test was also examined in duplicate titrations to obtain a measure of the accuracy of this method. Ten tests were performed using the same B/Lee virus preparation which had been used for the investigation of the pattern haemagglutinin test, and a primary dilution of 1/2 in calcium saline was used for half the titrations. The results shown in Table II-2 are the means of 2 readings

TABLE II - 2

REPRODUCIBILITY OF ENZYME TEST

Enzyme "units" of a B/Lee virus suspension. Tests performed as duplicate titrations with 2 primary concentrations of virus.

UNDILUTED		1/2 DILUTION	
INDIVIDUAL TITRATIONS	MEANS OF DUPLICATE TITRATIONS	INDIVIDUAL TITRATIONS	MEANS OF DUPLICATE TITRATIONS ϕ
270 272	271	140 140	280
280 284	282	133 130	266
273 278	275.5	134 134	268
276 276	276	134 144	278
272 278	275	131 141	272

ϕ Multiplied by 2

made on individual samples in the spectrophotometer, and from which the mean readings for the blanks have been subtracted. All corrected optical densities were then multiplied by 100 to convert them to enzyme units, and the units for the diluted virus were multiplied by 2 for comparison of the means of duplicate titrations. The standard error of the mean of duplicate titrations was 1.86%, which compared favourably with the standard errors of both haemagglutinin titrations.

COMPARISON OF TITRATION METHODS WITH DIFFERENT VACCINES

Since 3 simple methods for the approximation of the viral content of influenza vaccines were shown to be reasonably accurate and reproducible, the relationship between the titres obtained with different types of test was investigated to discover if the methods might be used interchangeably with all virus strains and vaccine preparations.

For this experiment virus suspensions of 3 different strains were prepared as described under Methods and Materials, and treated with physical or chemical agents which are known to inactivate infectivity of the virus for mouse lungs and the allantois of chick embryos. Details of the methods of inactivation may be found in Appendix 1. The B/Lee virus suspension, which had been stored at $4^{\circ}C$ for 3 months, was found to be completely non-infectious in either of the test systems, prior to treatment with inactivating agents. Virus suspensions of A/Swine and A2/Singapore/1/57M still

TABLE II - 3

COMPARISON OF CCA, PATTERN, AND ENZYME TESTS

Titres of vaccine preparations measured by the CCA test of Miller and Stanley (1944), a pattern haemagglutination test (Fazekas de St. Groth and Graham,1955), and a test based on the measurement of sialidase activity (see text).

VIRUS STRAIN	VACCINE PREPARATION	METHOD OF TITRATION			RATIO CCA/PATTERN TITRE
		ENZYME UNITS	CCA UNITS	PATTERN RECIPROCAL TITRE	
B/Lee	Untreated	265	1764	53700	1:30
	Ultra-violet Irradiation	273	1782	46770	1:26
	Sulphur Mustard	250	1646	46770	1:28
	Formaldehyde	270	2024	46770	1:23
	Heat	42	1092	38020	1:35
	Mercurial	86	1871	38020	1:20
A/Swine	Untreated	137	1444	17780	1:12
	Ultra-violet Irradiation	122	1336	15140	1:11
	Sulphur Mustard	118	1482	17780	1:12
	Formaldehyde	106	N.D.	17780	N.D.
	Heat	40	994	17780	1:18
	Mercurial	70	405	10230	1:25
A2/Sing. 1/57M	Untreated	405	N.D.	7943	N.D.
	Ultra-violet Irradiation	406	N.D.	7413	N.D.
	Sulphur Mustard	315	N.D.	7943	N.D.
	Formaldehyde	396	460	7943	1:14
	Heat	127	390	6370	1:16
	Mercurial	388	542	6457	1:12

All results are the means of duplicate titrations. The standard errors of CCA, pattern, and enzyme tests were estimated at 4.12, 3.89, and 1.86% of the mean, respectively. Enzyme units were adjusted to each strain. Inter-strain comparisons of enzyme activity are not valid.

N.D. Not done.

retained considerable infectivity despite storage, and elimination of this activity by the various treatments was demonstrated before proceeding with the experiment.

All vaccines were then titrated by the enzyme and by the CCA and pattern haemagglutination tests. For each strain all the experiments were performed on the same day, and each titration was carried out in duplicate; the results are shown in Table III-3. As was to be expected, abolition of infectivity by heat treatment also destroyed most of the enzyme activity, but inactivation with a mercurial only affected the enzyme activity of the B/Lee and A/Swine vaccines. Other methods of inactivation appear to have influenced the enzyme titre to varying degrees with different strains.

The enzyme assays were performed on different primary dilutions with each strain, and inter-strain comparisons would not be valid. In any case, the enzyme method was precluded from further use as an assay system because it was proposed to use heat and mercurial inactivated vaccines in the immunogenicity studies. In some cases the treatments have had drastic effects on the haemagglutinin titres, more especially with the CCA test. There is overall evidence of a marked difference between the B/Lee and A/Swine strains in CCA/pattern test ratios. This indicates that these methods do not measure the same viral characteristics, and selection of the more suitable test for immunogenicity studies

would depend on demonstration of a closer relationship between test titre and immunogen content with one or other of the methods.

DISCUSSION

The three methods of assay which were examined were found to be reasonably accurate and reproducible, but the measurements arrivedat in each case did not show correspondence when studied with different virus strains and different methods of inactivation of vaccines. Problems associated with inter-laboratory variation, not studied here, have been observed with each of the methods, and have led to investigations by a number of workers. Most recently, photometric titrations have been the subject of a critical examination by Drescher (1957), who showed that variability of fowl erythrocytes was the major factor limiting the accuracy of the absolute titres with these particular methods. Drescher (1959) developed a highly accurate method for the photometric titration of haemagglutinating viruses, which Drescher et al. (1962) proposed as the test to be adopted for use on an international basis as a standard measurement of haemagglutinin. Their evidence showed that there was remarkable agreement between the results obtained by a number of different laboratories, using this Haemagglutination Concentration Unit (HCU) method of titration. However, an experiment in which they compared HCU and pattern test titres showed an even greater variation in the ratios of the two tests with different strains than was found in the work reported in this chapter of the thesis.

The explanation for these varying ratios is to be found in the different avidities of the viruses for fowl erythrocytes (McLean,1961), which result in easily observable differences in size of the cell aggregates in photometric titrations (Warburton, 1961). Where the clumps of cells formed are small, the sedimentation process may be too slow to permit estimation, or even detection, of virus. Levine et al. (1953) endeavoured to overcome this difficulty by using low concentrations of virus to ensure that only dimers of red cells were formed. Their photometric method and the similar method of Horsfall (1954), as a consequence, required over two hours settling, during which period fluctuations in ambient temperature might well introduce another large source of variation. Isaacs (1957) has provided an explanation for the insensitivity of these methods, and pointed out the difficulties which would arise if they were used for the assay of suspensions containing virus filaments.

Photometric tests in general have been found to be less sensitive to the presence of virus than pattern tests; the latter are therefore to be preferred as a means of approximating virus content of vaccines. More importantly, McLean (1961) observed that in the course of adaptation of human influenza viruses to eggs in 1957, pattern test titres rose more rapidly than CCA titres, and antigenicity of suspensions of these viruses corresponded to the pattern test titres. He also observed that certain so-called "high lines" of PR8 virus gave much increased CCA titres, but were less antigenic in

both mice and men on a unit basis than other passage lines of the same strain with average CCA capacity. It would appear therefore that pattern test titres are more satisfactory as rough measures of immunogen content, and the method of Fazekas de St. Groth and Graham (1955), described above, was used in the work to be described in succeeding chapters.

SUMMARY

1) Three methods for approximation of the virus content of influenza vaccines were found to be reasonably precise and reproducible.

2) Virus enzyme was inactivated to varying degrees by different agents which were used for the destruction of infectivity in the preparation of vaccines. This precluded the use of enzyme tests as assays of vaccine for immunogenicity studies.

3) The CCA photometric and a pattern haemagglutinin test were shown to measure different viral characteristics. Pattern tests were preferred for studies in succeeding chapters because other workers have shown that they are more sensitive assay systems, and give a more direct measure of antigen content.

CHAPTER 3

EXPERIMENTAL INFLUENZA IN THE LABORATORY MOUSE

INTRODUCTION

The work reported in this thesis was planned with the intention of studying the immunogenicity of influenza virus vaccines in a model system. In this way it was hoped that fundamental principles might be disclosed which would subsequently be found to have general application to immunity in influenza. The choice of subjects for this model system could not be the natural hosts - men - since the almost universal experience of humans with this disease renders them unsuitable for studies with familiar strains, and there would be danger in using viruses such as human adapted fowl plague (Pereira, 1960). It was, therefore, necessary to select a species of laboratory animal which could be used for these studies.

In selecting an experimental animal, two criteria had to be kept in mind. Firstly, the animal would have to show symptoms typical of influenza following infection by the respiratory route, and secondly, be available in sufficient numbers to permit large scale investigations.

Consideration of the animals available for experimental work in Australia shows that mice, ferrets, and cynomolgus monkeys all satisfy the first criterion, but only mice can be obtained in large numbers. Andrewes et al. (1934) observed that influenza viruses may produce a characteristic form of consolidation in the mouse lung which has subsequently been the subject of extensive experimental work. Hers et al. (1962) have studied the histopathology and cytopathology of influenza virus pneumonia by means of fluorescent

antibody staining. Their work shows that the experimental virus pneumonia in mice is basically the same process as that seen in humans (Hers and Mulder, 1961). Strains of influenza virus can be adapted more or less readily to growth in mouse lungs, and the process may be facilitated by the use of cold environments (Briody et al. 1953) or certain intranasal adjuvants (Jones, 1950). The strain A/Swine/1574 of Shope (1935), which he found did not require adaptation, was used in many of the experiments which will be reported below.

Another point in favour of the use of mice in such studies is that there are well developed techniques available both for challenge and for assessment of the outcome of infection. The standard intranasal method of inoculation was chosen for challenges of the mice. In the hands of an experienced operator, using careful anaesthesia, with a mixture of ether and chloroform, this method has been shown to result in delivery into the lungs of a volume of inoculum which varies within very narrow limits (Fazekas de St. Groth, 1948). Assessment of the results at autopsy was based on a modification of the Horsfall (1939) method, in which observations of the time of specific death, and also degrees of pulmonary consolidation in mice surviving for seven days, are allotted numerical values (Mouse Scores) ranging from 5.0 for death on the 3rd or 4th day, down to 0.1 for minimal evidence of lung consolidation. Quantitative interpretation of responses by means of such indices provides assay systems which may be referred to as "semi-quantal" (Finney, 1952). In the region of median responses these yield much more precise estimates of the

correlation between dose and response than can be obtained with quantal assays. The 50% end-points for influenza virus infection in mice correspond to mouse scores of 2.5, and Lauffer and Miller (1944), using a large number of mice, demonstrated that calculations of such end-points were also more reproducible than $L.D._{50}$ estimations based on a quantal assay involving death or survival on the 10th day. In the work to be described below, the numerical value applied to day of death or extent of lung lesions will be designated the "mouse score" or M.S. Details of the intranasal technique used, and method of interpretation of results, are given in Appendix 1.

It is a fundamental principle of biological assay that factors, other than dose, which might affect the response, should be eliminated as far as possible, and that those which cannot be eliminated must be kept constant for all the test subjects (Jerne and Wood, 1949).

The work to be described in this chapter was designed to discover, or to re-examine, those factors which influence the dose response curve in the challenge test with which it was proposed to measure immunity levels in mice. So far as intranasal challenge with influenza virus is concerned, some of these factors have already been studied by others, and it will merely be necessary to outline the results which they have obtained, and the methods adopted to eliminate their influence. Some will be examined as individual problems, and others, such as age, weight, and sex, where

there may be interactions, will be considered in conjunction with one another.

FACTORS INFLUENCING THE OUTCOME OF INFLUENZA IN MICE

Factors that may influence the outcome of influenze in mice can be grouped under 3 broad headings, metabolic factors, host factors, and environmental factors. Metabolic factors include nutritional status, water supply, and oxygen tension; host factors that may influence the outcome of influenza in mice are strain, age, sex, and weight of the animals; and environmental factors that must be considered in studies of this type include ambient temperature, humidity, and barometric pressure.

A. Metabolic Factors

1) Nutritional Status:- Saslaw et al (1946) reported that monkeys (Macaca mulatta) became much more susceptible to intranasally administered influenza when they were maintained on deficient diets. Sprunt (1948) found that mice held on a low protein diet showed a greatly increased mortality from intranasal influenza virus if given injections of methionine. Although Kalter (1949) could not confirm this result, Sprunt and Flanigan (1956) further investigated the effect of maintenance on a protein deficient diet in mice on the mortality from a standard dose of influenza virus. Two weeks on a low protein diet appeared to result in an increased mortality; but if mice were kept on the deficient diet for a longer period they became less susceptible to the disease. After eight weeks deficiency the test mice appeared to be more affected by the virus once more,

TABLE III - 1.

ADEQUACY OF DIET

Mean Weights (g.) of 286 female W.E.H. mice at stated ages, showing the weekly increase in weights.

Age in weeks	8	9	10	11	12	13
Weight	32.85	33.27	33.78	34.28	34.66	35.01
Increase	-	0.42	0.51	0.50	0.38	0.35
Age in weeks	14	15	16	17	18	19
Weight	35.31	35.56	35.81	36.12	36.33	36.54
Increase	0.30	0.25	0.25	0.31	0.21	0.21
Age in weeks	20	21	22	23	24	
Weight	36.74	36.92	37.00	37.12	37.21	
Increase	0.20	0.18	0.08	0.12	0.09	

and the experiment was not carried beyond that point. The published data indicate that the 815 control mice showed a slight drop in mean weight over the period of test, indicating that the standard diet was less than adequate. However, the 794 mice under test lost an average 3.375 g. as a result of the "restricted" diet, and the authors' conclusions that the ultimate outcome of influenza virus infection in protein deficient mice varies with the extent of the protein depletion, may be correct.

In this present study, the probable effect of diet on the dose response curve was minimized by providing an excess of food at all times. The protein and fat content of the cube and pellet type food used were respectively 21.7% and 5.1%. The level of food in the tins was checked twice a day, and to test the fundamental adequacy of this diet 286 Hall Institute (W.E.H.) random bred female mice were weighed weekly from the age of eight weeks until they were used in a challenge test at the age of 24 weeks. The results are shown in Table III - 1. At each weekly weighing there was an increase in mean weight, as would be predicted in the case of an adequate diet.

2) Water : - No previous experimental work could be found which had been carried out to ascertain the effect of dehydration on experimental influenza infection in mice, but it was a reasonable assumption that availability of water could be a factor to consider. As yet there does not appear to have been a basic study of the role of water in experimental laboratory infections. McLaren (1961) found that restriction of the water supply affected the internal temperature of

mice, and Chew and Hinegardner (1957) studied the effect of chronic insufficiency of drinking water on food intake and body weight of adult mice. Even moderate restriction resulted in a reduction of food consumption, and body weight was barely maintained.

Two special cages were available in which it was possible to restrict the supply of water to mice. These were used in an experiment designed to discover the effect of moderate dehydration on the growth rate and on the antibody response to influenza vaccines of W.E.H. mice. Subsequently the resistance of these immunized mice to challenge was also tested. The results of these experiments indicate that restriction of the water supply had a greater effect on the susceptibility of mice to infection than on the antibody response. The experiment is therefore reported here, rather than in the chapter devoted to the study of the antibody response of mice to vaccination.

Effect of restricted water supply on weight, antibody production, and resistance to challenge of immunized mice

Twelve male W.E.H. mice were weighed, and transferred to tins with restricted access to water so that the animals drank approximately 50% of the intake of controls given unlimited access. At the same time, from a large number of male mice of the same age, twelve animals were selected whose individual weights corresponded exactly to those of the first group. These were placed in two cages with an unrestricted water supply, and all mice were allowed free access to food. After seven days for "stabilization",

TABLE III - 2

Effect of dehydration on the weight gain, antibody response, and resistance to challenge of W.E.H. mice

Individual body weights, serum anti-haemagglutinin titres and responses to challenge (M.S.) of twelve mice of corresponding initial weights, allowed restricted and free access to water respectively.

Initial Weights (g.) 8 week-old mice	Final weights (g.) 12 week-old mice		Serum anti-haemagglutinin Titres $\log_{10}$		Outcome of Challenge (M.S.)
	Access to water		Access to water		Access to
	Restricted	Unrestricted	Restricted	Unrestricted	Restricted
34	36	42	2.04	1.66	0.3
34	34	40	1.42	1.30	4.5
32	30	36	1.75	2.15	2.0
32	35	40	2.15	1.60	2.5
30	31	36	1.45	1.30	5.0
30	32	34	1.36	1.45	4.5
28	27	38	2.24	1.96	1.8
28	31	39	1.36	2.21	4.5
26	27	33	1.54	2.18	1.0
26	29	31	1.63	1.27	4.0
24	24	30	1.45	1.90	4.5
24	26	32	1.00	1.84	5.0
Means 29.00	30.17	35.92	1.610	1.735	3.30

Death from influenza infection on or before the 7th day post-inoculation.

Water
Unrestricted
1.5
2.8
0.8
2.5
3.3
4.5
1.5
1.3
0.5
4.5
4.0
0.8
2.33

all mice were inoculated intraperitoneally with 0.25 ml. of an A/Swine vaccine, inactivated with 0.025% formalin, which contained 440 pattern haemagglutinin units per ml.

Nineteen days after immunization, all mice were bled by the retro-orbital technique of Halpern and Pacaud (1951). The sera were separated and titrated for anti-haemagglutinin content, after treatment with Receptor Destroying Enzyme and duplicate absorptions with fowl cells to remove the natural antibodies to these cells which mouse sera possess in a marked degree (see Chapter IV). Details of serum treatment and the method of anti-haemagglutinin testing are in Appendix 1. Two days later the "restricted" mice were allowed unlimited access to water for four hours, and then both groups of animals were weighed. They were challenged intranasally with a dose of A/Swine virus shown by previous titration to contain 100 M.S. 2.5 and then returned to the appropriate cages. The experiment was terminated 7 days later, when all surviving mice were autopsied.

Results are given in Table III-2 and the severe effect of dehydration on body weight gain is clearly demonstrated. Although there is no evidence of a reduction in the antibody response, there is however an indication of increased severity in the reaction to challenge, in that there was a greater mortality in the restricted mice. The overall difference between the groups is not statistically significant, but the trend is sufficiently large to indicate the importance of maintaining test mice in circumstances which allow them

free access to water.

This factor was also controlled by the twice daily check of all animals. Water bottles were examined not only to see that excess was always available, but that the mice were drinking a sufficient quantity each day. It was noted that on one occasion when mice in one tin became ill and had to be rejected, the first indication of sickness was an untouched water bottle.

3) Oxygen Tension:- Berry et al. (1955) found that lowering the atmospheric pressure reduced the mortality and extent of lung consolidation in PR8 infected mice. Kalter et al. (1955) found that this was associated with a reduced rate of virus growth in the lungs, although the titres finally attained in these organs were the same as those from the lungs of infected mice held in a normal atmosphere. From these two reports it is not possible to decide whether the effects observed were due to hypoxia resulting directly from lowered oxygen tension, or to the overall effect of reduction in atmospheric pressure.

The better controlled study of Sawicki et al (1961) showed that a raised oxygen tension increased the mortality, and lowered the survival time, of mice infected with either type A or type B virus. From their work it is clear that the level of oxygen in the atmosphere may affect the outcome of influenza infections in mice. To control this factor mice were always held in well aired rooms in reasonably open containers.

In summary it can be stated that food supply and the level of oxygen tension have a fundamental bearing on the outcome of influenza infection in mice. In the light of its influence on overall food intake, and the evidence presented here, water supply must also be assumed to be of importance. The effects of these three factors were therefore kept constant for all test animals by providing unrestricted access to fresh water and an adequate diet, and by proper design of accommodation for the mice.

B. Host Factors

In the past a variety of host factors have been shown to influence the outcome of infections in mammalian species. In the present work, variations in strain, age, sex and body-weight were selected for study as factors which might affect the response of mice to intranasal challenge with influenza virus.

1) Strain of Mice :- de Gara and Furth (1945) observed that agouti (C3H) inbred mice suffered a higher morbidity and mortality than several Swiss inbred lines when exposed to type B influenza virus. Briody et al. (1953) first highlighted the variable facility with which different lines of mice "adapt" human influenza viruses. Recently Lindenmann (1962), in a preliminary report, has described the complete insusceptibility of A_2G mice to massive intracerebral challenge with N.W.S. virus, and reduced damage to the lungs of this line compared with random bred albino mice, when intranasal challenge with the parent non-neurotropic WS virus was used.

From the variety of mouse strains which were available, the random bred Hall Institute (W.E.H.) and the inbred C57Bl were chosen for use in the proposed studies on immunogenicity of influenza virus vaccines. The former were used by Fazekas de St. Groth and coworkers in a lengthy investigation of immunity to influenza in mice (1950, 1951, 1954), and the latter were used in experiments on antibody production (Fink and Quinn, 1953; Ipsen, 1954), and in a study of the mechanism of resistance to ectromelia virus (Schell, 1960). Consideration of the genetic background of these strains indicated that their responses to influenza infection might be different. Accordingly an experiment was set up to compare the outcome of infection in the two strains, using a range of challenge doses of virus.

Response of C57Bl and W.E.H. mice to influenza virus infection

Female C57Bl and W.E.H. mice, 16 and 19 weeks old animals of each strain, which had been segregated since weaning, were allocated to cages, according to ages, in groups of 5, using a table of random numbers (Fisher and Yates, 1957). In this and subsequent experiments using mice a number was allotted to each animal, and for purposes of identification the mice were subjected to ear lobe cutting, each cut being regarded as having a value of a power of 2. The process of identification was intended as a precaution against accidental mixing of animals on test, and to permit experiments designed to test correlations of antibody and challenge responses with body-weights of animals.

Results of Analysis of Variance of Age and Strain of Mice

Source of Variation	Significance
Dose	***
Age	N.S.
Strain	N.S.
Dose-Age	N.S.
Dose-Strain	*
Age-Strain	N.S.

Levels of significance are denoted: * 5% level
** 1% level, and
*** 0.1% level
N.S. Not significant

TABLE III - 3

Responses of C57Bl and W.E.H. mice to Influenza Virus Infection

Average M.S. of group of five female mice challenged with stock A/Swine virus.ϕ

Dilution of Challenge Virus	W.E.H.Strain: Age in Weeks 16	W.E.H.Strain: Age in Weeks 19	W.E.H.Strain: Overall Means for Strain	C57Bl Strain: Age in Weeks 16	C57Bl Strain: Age in Weeks 19	C57Bl Strain: Overall Means for Strain
10^{-2}	-	3.90	-	-	4.125	-
10^{-3}	2.30	2.40	2.35	3.80	2.90	3.35
10^{-4}	2.66	2.06	2.36	1.32	2.52	1.92
10^{-5}	0.82	1.22	1.02	0.88*	0.48	0.68
10^{-6}	1.00	0.82	0.91	0.82	0.80	0.81
10^{-7}	0.44	0.70*	0.57	0.16	0.32	0.24

ϕ Undiluted stock A/Swine virus contained $10^{6.05}$ ID_{50} per 0.05 ml. in allantois-on-shell (Fazekas de St. Groth and White,1958).

* One missing observation, mean M.S. of four mice.

A small sample of blood was obtained from each mouse, using the retro-orbital technique, and absence of anti-haemagglutinin antibody to A/Swine virus from all sera was demonstrated by the technique described in the Appendix. All sera were treated with Receptor Destroying Enzyme to remove non-specific inhibitors, and were twice absorbed with packed fowl cells.

The mice were challenged intranasally with graded doses of stock A/Swine challenge virus suspended in standard medium, 5 mice to each dose level, and infectivity tests in surviving allantois on shell (Fazekas de St. Groth and White, 1958) were carried out on the lowest dilution of challenge virus (10^{-2}) both prior to and at the conclusion of this challenge test in mice, and in all subsequent challenge tests reported in this chapter. Specific deaths and extent of lung lesions at autopsy, were used to estimate Mouse Scores, (M.S.) as before. The means for each dose level and age are presented in Table III - 3, which also shows the overall means for each strain. The data for individual mice may be found in Appendix 2, Table A, and analyses of variance comparing dose, age and strain for the five higher dilutions in both age groups, and dose and strain for 19 weeks old animals only, are given in detail in Table B of the same Appendix. Insufficient 16 weeks old mice were available to allow analysis of both ages at all dose levels.

The analyses indicate that there was no significant difference in the overall responses of the two strains, at either age level or with the combined ages. Inspection of the mean M.S. shown

in Table III - 3 suggests that the slope of the response of C57Bl mice was steeper than that of W.E.H. mice, and there is some confirmation for this in the analysis of the higher dilutions in both ages. Here the dose-strain interaction is shown to be just significant at the 5% level. With a difference in slopes of minor degree however, there was no necessity to abandon the use of the more plentiful and far more robust random bred W.E.H. strain in favour of C57Bl mice.

2) Age of Mice : - The influence of age on susceptibility to infections has often been reported; Sigel (1952) has reviewed the literature relating to virus diseases, with particular reference to laboratory animals. Kalter (1949) studied the outcome of infection with A/PR8 virus in Swiss mice at seven ages ranging from 3 to 30 weeks. With increasing age, these mice required increasing amounts of inoculum to produce a specified mortality, or degree of lung damage. Decreasing levels of susceptibility were even observed in the clearly adult ages between 13 and 21 weeks; but although the LD_{50} for 30 weeks old was at least 1000-fold greater than for 3 weeks old mice, there appeared to be no significant difference between the amounts of virus in the lungs of both groups at 48 hours, with each of two widely spaced challenge doses. The older mice had a longer "eclipse" phase than the younger, and also a lower rate of virus proliferation.

In the experiment reported earlier in this chapter which

investigated the influence of strain of mice on the response to A/Swine infection, challenge was carried out at two age levels. Table III - 3 shows no evidence of an age effect, and the analysis of variance of the results (Appendix 2, Table B) indicated nearly identical overall responses at the two age levels in both strains of mice. Since this was not in accord with the earlier work of Kalter it was necessary to conduct a further investigation into age of mice as a possible factor influencing the outcome of influenza in mice. Age was combined with sex and weight of animal in a factorial experiment which will be reported below.

3) Sex of mice: - Sex has also long been recognised as a factor which influences the outcome of infections, and early work was reviewed by Perla and Marmorston (1941). Recently, Hurst et al. (1960) have reported finding a greater mortality in male mice as a result of intramuscular inoculation of equine encephalomyelitis virus, and this was apparently due to the development of lower levels of viraemia in the blood and brain of the female.

In the experimental work described in this Thesis, only sexually mature mice (at least 6 to 7 weeks old) were used, since Kalter (1951) found that castration diminished, and the administration of testosterone increased, the rate of A/PR8 virus multiplication in Swiss mice.

4)Weight of mice : - In experimentation designed to study the relationship between dose of some test material and response in

animals, it is customary to use only subjects with identical body weights. This precaution is often taken without any evidence of a real connection between body-weight and host response in the particular test system. Where a correlation has been shown to exist, as in the case of meningococcal infection of mice, (Pittmann, 1941), equal precision may often be gained by using mice of less uniform weight, and eliminating effects due to this factor by means of analysis of covariance (Fisher, 1948). More importantly, however, body-weight varies with both age and sex of mice, and it is essential to eliminate its influence before drawing conclusions as to the significance of these other factors. It was therefore decided to study age and sex of mice in a factorial experiment with weight as a covariate of both.

Influence of age, sex and weight of mice on the outcome of influenza virus infection in mice

Groups of 60 W.E.H. mice, approximately equal numbers of each sex, were obtained at intervals of 3 weeks, until 5 ages had been accumulated. The mice were segregated immediately after weaning, and were constantly checked until the youngest group were 10 weeks old. Each animal was then numbered and identified, and weighing was carried out as quickly as possible, using the technique described in Appendix 1. There is, however, a large diurnal variation in the weights of individual mice, and the animals used in this experiment were found to have a variation of 3 to 8 % of their mean body-weight over a period of nine (daylight) hours. Consequently,

although weighings were always carried out to the nearest gram, the operation of weighing 300 mice took some hours and mice were therefore regarded as belonging to 5 weight "classes" , to which arbitrary numbers were assigned for the purposes of statistical analysis of the results of the experiment. Selection of the range of weights for each class was based on the observation that histograms of the weights of W.E.H. mice were, with only minor discrepancies, always clearly normal distributions. The overall distribution of weights of mice to be used in this particular experiment had a mean of 35.9 g. and a standard deviation of 5.34 g. Accordingly, mice with weights less than 31 g. (approximately 1 standard deviation below the mean) were allotted to class 1, those above 42 g. to class 5, and those within one standard deviation of the mean, above and below, were allotted to classes 2, 3 and 4, according to increasing weight. There were approximately equal numbers in each class.

Preliminary blood sampling and testing for anti-haemagglutinin was carried out on all animals, and antibody to A/Swine virus could not be detected. Two days after bleeding , mice of each age and sex were allocated to 6 groups of 5 animals, and proper randomization was again performed with the aid of random numbers. A challenge test was then carried out, using tenfold dilutions of the stock A/Swine virus in standard medium for the intranasal inoculation of the mice, and animals of each age of both sexes were tested with the full range of challenge doses. Mouse

TABLE III - 4

Variation in response with Sex and Age of W.E.H. Mice

Average mouse scores (M.S.) of groups of five mice challenged with stock A/Swine virus at stated dose levels.

Dilution of Challenge Virus	Females Age in Weeks				Males Age in Weeks				
	10	13	16	19	10	13	16	19	22
10^{-3}	2.80	3.04	2.30	2.40	2.46	2.66	2.06	2.20	2.44
10^{-4}	2.86	2.06	2.66	2.06	1.72	1.64	1.38 ϕ	1.36	1.28
10^{-5}	0.96	0.66	0.82	1.22	1.20	1.26	1.26	0.98	0.96
10^{-6}	0.66	0.72	1.00	0.82	0.56	0.55 ϕ	0.90	0.86	0.62
10^{-7}	0.30 ϕ	0.24	0.44	0.70 ϕ	0.38	0.23 ϕ	0.18	0.54	0.14
10^{-8}	0.00	0.06	0.02	*	0.02	0.00	0.08	0.12	0.02 ϕ

*Missing block

ϕ One missing observation, mean M.S. of four mice.

Challenge with A/Swine Virus performed by means of the technique described under intranasal challenge in Appendix 1.

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Results of Analysis of Covariance - Age and Sex with Weight as Cofactor

Source of Variation	Significance
Sex	*
Age	N.S.
Dose	***
Age - Sex	N.S.
Sex - Dose	N.S.
Age - Dose	*

Levels of significance are denoted as in Table III - 3

scores were estimated after seven days, and the means for each dose level are shown in Table III - 4. No data are presented for 22 weeks old females, because of a large number of accidental deaths in these mice. The individual results along with the weight class of each mouse are given in Table C of Appendix 2.

The individual mouse scores were submitted to analysis of covariance, with weight class treated as cofactor. The analysis followed a method employed by Yates (1934) which is outlined by Quenouille (1952), in which the interaction component of each variant with the cofactor is subtracted before the variance is estimated. In this type of analysis the sum of squares corresponding to each source must be combined with the residual sum of squares before the effects of the cofactor are eliminated.

Missing observations were replaced by the mean for the four remaining observations, and the data appropriate to the highest dilution (10^{-8}) were not used, so that the missing block at that level did not have to be considered. Data for 22 weeks old males, of course, also had to be omitted, and only four age levels of mice were included in the analysis.

Details of the analysis are shown in Appendix 2, Table D, and it is obvious that weight or age of the host did not influence the result of the challenge test with A/Swine virus in W.E.H. mice. This was an unexpected result in view of the extreme "age" effect found by Kalter (1949) with A/PR8 virus challenge in a

commercial line of Swiss mice. However, inspection of the results for the 5 age levels of male mice, shown in Table III - 4 provides confirmation that age of mouse was not a factor which influenced the outcome of A/Swine virus infection in these animals.

The analysis indicates that sex might be a factor which could influence the results of challenge, and that the slope of the dose-response curve might be different with male and female mice. The variance ratios for sex and sex-dose interaction are at the borderline of 5% levels of significance, however, and no definite statements may be made on this evidence.

Sex was therefore examined in other experiments designed to study the possible effect of this factor in more detail.

Influence of sex on the outcome of influenza virus infection in mice

A series of experiments were carried out to examine the effect of sex of mice on the response to challenge with graded doses of a variety of strains of influenza virus. In the first experiment, a further investigation was made of the influence of sex on challenge with A/Swine virus, using an increased number of male and female mice at each dose level.

A large group of 8 weeks old W.E.H. mice of both sexes were weighed, and female ranging in weight from 21 to 28 g. and males from 23 to 30 g. were selected. These were then distributed, according to sex, in cages in lots of five, so that the mean weights

TABLE III - 5

Investigation of sex as a factor which might influence intra-nasal challenge with A/Swine virus.

Average M.S. of 15 male and 15 female W.E.H. mice, challenged with graded doses of A/Swine virus. 8 weeks old mice.

Dilutions of Challenge Virus	Sex of Mice	
	Male	Female
10^{-2}	4.41	4.76
10^{-3}	3.71	3.73
10^{-4}	2.76	2.77
10^{-5}	1.76	1.77
10^{-6}	0.95	0.59

TABLE III - 6

Enhanced effect of B/Lee virus in male W.E.H. mice

Average M.S. of 15 male and 15 female W.E.H. mice, challenged with graded doses of stock B/Lee virus, 8 weeks old mice.*

Dilutions of Challenge Virus	Sex of Mice	
	Male	Female
10^{-2}	3.01	2.31
10^{-3}	2.11	1.37
10^{-4}	1.01	0.66
10^{-5}	0.89	0.44
10^{-6}	0.18	0.17

* Undiluted stock B/Lee virus contained $10^{6.2}$ LD_{50} per 0.05 ml. in allantois-on-shell.

of mice in all cages were approximately 25 g. for females, and 27 g. for males. Suitable 10-fold dilutions of stock A/Swine virus were prepared in standard medium, and were inoculated intranasally into 15 mice - three cages - of each sex, at each dose level. Specific deaths and the extent of lung lesions at autopsy were used to estimate the mouse scores, and the means for 15 mice at each dose level are presented in Table III - 5. The results clearly show that sex of mice did not influence the outcome of challenge with A/Swine virus.

Consideration was then given to the possibility that the sex of the host might influence the outcome of infection with other types and strains of influenza virus. The previous experiment was therefore repeated again using 8 weeks old mice with the same range of weights, but employing stock B/Lee as the challenge virus. Dilutions were again prepared in standard medium and inoculated intranasally into 15 male and female mice at each dose level. The mean M.S. of the two sexes at each dose level are compared in Table III - 6, and a large difference in response between the sexes is immediately apparent. Confirmation of the increased severity of reaction to challenge with B/Lee virus in male mice was obtained with the same range of doses in small numbers of 28 weeks old W.E.H. mice. Again there was a large difference between the response of the sexes at each dose level. However, the challenge doses for both experiments were made up in standard medium which contains chloromycetin, and it was possible that this antibiotic had influenced the results. Hurst et al. (1960) reported that mepacrine

TABLE III - 7

Variation in responses with Sex in C57Bl mice. Challenge with B/Lee virus

Average M.S. at each dose level in 2 experiments using equal numbers of male and female mice.

		Dilution of stock challenge virus				
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Experiment No. 1 ϕ	Male		4.63	1.23	0.78	0.25
	Female		1.63	0.80	0.70	0.20
Experiment No. 2 *	Male	5.00	4.20	2.60	1.06	
	Female	3.83	2.30	1.40	0.98	

ϕ 5 mice, 12 weeks old, of each sex at each dose level.

* 8 mice, 16 weeks old, of each sex at each dose level.

enhanced the variation in susceptibility of the sexes to infection with equine encephalomyelitis virus in mice, and the interaction of anti-metabolite drugs with the host may influence the outcome of all experiments of this type. Further experiments were therefore performed to study the outcome of challenge with B/Lee virus in both sexes of mice, using a simple salt solution, calcium magnesium saline (see Appendix 1), as a suspension medium for the challenge doses. The C57Bl strain of mice were used in these experiments to investigate the additional possibility that the differential response of the sexes might have been peculiar to the W.E.H. strain.

Investigation of the enhanced effect of B/Lee virus in male mice

Limited numbers of C57Bl mice of two ages were available, and were employed in experiments to study the effects of B/Lee virus in male and female animals. In the first experiment five male and five female 12 weeks old mice and in the second, eight male and eight female 16 weeks old mice were challenged at each dose level. No particular system was required in the distribution of these inbred mice, because all the males weighed approximately 25 g. and all the females approximately 22 g. The challenge dose levels chosen covered the same range as in the previous experiments, except that there were insufficient mice to include all doses in each experiment. All dilutions were made in calcium magnesium saline, and the outcome of intranasal challenge was followed, as previously, for 7 days. Mean M.S. were calculated for each dose level and sex in both experiments and the results are shown in Table III - 7. A considerable difference

TABLE III - 8

Responses of male and female mice to B/Tas/27/53M[ϕ] challenge

Average M.S. of W.E.H. mice at each dose level in 2 experiments using equal numbers of male and female mice.

	Sex	No. per dose	Dilutions of challenge virus 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Experiment No.1	Male	8	-	3.42	3.22	1.75	1.01	0.15
	Female	8	-	3.80	2.60	1.64	1.16	0.21
Experiment No.2	Male	7	4.50	3.40	2.38	1.62	-	-
	Female	7	4.18	3.80	2.45	1.55	-	-

ϕ Undiluted B/Tas/27/53M challenge virus contained $10^{6.5}$ LD_{50} per 0.05 ml. in allantois-on-shell.

between the effects of B/Lee influenza virus infections in male and female mice is apparent.

Table III - 8 shows the results of two experiments designed to investigate the possibility that the enhanced effect of challenge in male mice was common to type B influenza virus strains. In these experiments 8 weeks old W.E.H. mice were allocated to cages so that each dose level contained mice of the same average weight for each sex. Female mice had a mean weight of 27 g. with maxima and minima of 23 and 29 g. Male mice, with a mean of 30 g. ranged in weight from 25 to 33 g. Challenge was carried out with a type B virus isolated in 1953 in embryonated eggs, and subsequently adapted to growth in the mouse lung. Dilutions of the virus were made in calcium magnesium saline, and challenge with graded doses was carried out as before. The results were approximately the same for males and females, indicating that differentiation of the outcome of infection according to sex was not common to all type B strains.

A similar experiment was performed using challenge with A/Bel influenza virus, a strain which grows to high titre in the allantoic cavity of embryonated eggs, but which causes only minimal lesions in the mouse lung, unless administered in an overwhelming dose. This test was planned to discover the effect of sex of mice on the outcome of challenge with virus of low virulence. Only a small number of 8 weeks old mice were tested at each dose level, and these were distributed as in the previous experiments. At the same time, duplicate challenge experiments of the same type were carried out

TABLE III - 9

Responses of male and female mice to challenge with A/Bel* influenza virus.

Average M. S. of mice at each dose level.

Sex	Number per dose	Dilutions of challenge virus			
		Undiluted	10^{-1}	10^{-2}	10^{-3}
Male	6	4.75	3.40	2.02	0.23
Female	6	4.25	3.83	1.45	0.78

*Undiluted A/Bel challenge virus contained $10^{8.06}$ ID_{50} per 0.05 ml. in allantois-on-shell.

TABLE III -9a

Responses of male and female mice to challenge with A2/Singapore/1/57M ϕ

Average M. S. of mice at each dose level

	Sex	Number per dose	Dilutions of challenge virus			
			10^{-1}	10^{-2}	10^{-3}	10^{-4}
Experiment No.1	Male	6	4.62	3.00	1.26	-
	Female	6	4.35	2.84	1.30	-
Experiment No.2	Male	5	5.00	3.25	0.88	0.25
	Female	5	5.00	3.38	0.42	0.20

C57Bl mice

ϕ Undiluted A2/Singapore/1/57M challenge virus contained $10^{5.78}$ ID_{50} per 0.05 ml. in allantois-on-shell.

using a mouse line of A2/Singapore/1/57, which still shows a large proportion of filamentous forms when stained by the technique of Lindenmann (1957). This strain of virus was tested in both 8 weeks old W.E.H. and 10 weeks old C57Bl mice; the former were again distributed evenly to each dose level on a body-weight basis, whilst the latter mice were of even weight and were distributed randomly. Male W.E.H. mice were, on the average, approximately 4 g. heavier than females, and C57Bl males were approximately 3 g. heavier than females.

In all three tests the viruses were diluted in calcium magnesium saline, and the challenge tests were performed according to the usual techniques. Mean M.S. for each lot of mice were calculated and are shown in Table III - 9 and Table III - 9a. There was no indication that sex of the mice had any influence on the outcome of infection.

In the studies of Hurst et al (1960), referred to above, it was noteworthy that the increased susceptibility of male mice to equine encephalomyelitis virus was accompanied by a 100 - fold greater level of viraemia in male than in female animals. Accordingly, it was decided to investigate the growth of B/Lee virus in the lungs of male and female mice, to determine if the increased consolidation produced in the lungs of the male animals over a wide range of doses was due to an increased rate of virus proliferation, or to the final attainment of a higher level of virus in male mice.

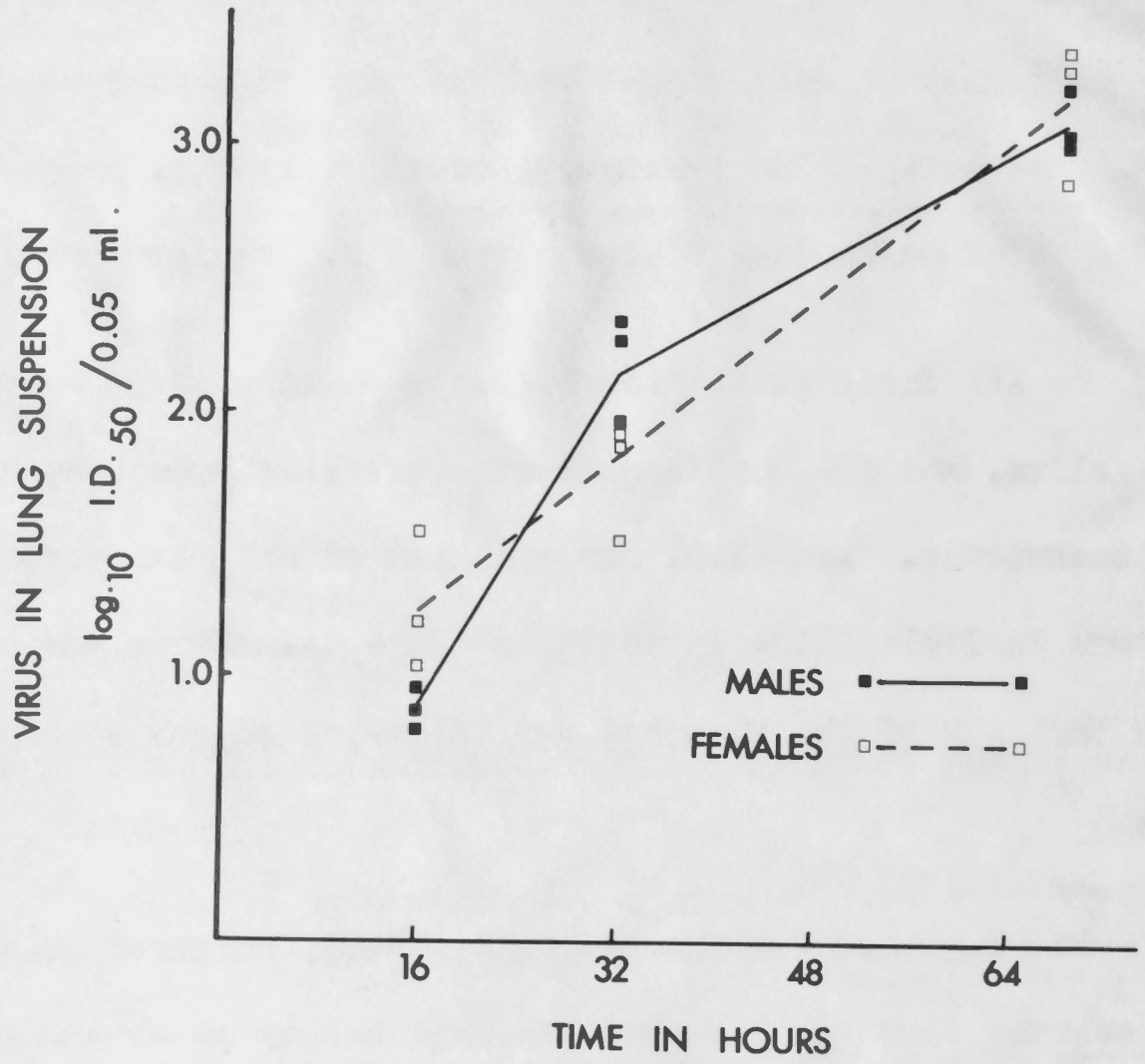


FIGURE III - 1

Growth of B/Lee virus in the lungs of male and female mice.

Males _____

Females - - - - - -

Investigation of the growth of B/Lee influenza virus in male and female mice

A study of the growth of this virus in mouse lungs was made, using the allantois-on-shell infectivity test of Fazekas de St. Groth and White (1958), to compare the production of infectious virus in the male and female mice. Fourteen W.E.H. mice, 10 weeks old, of each sex, and acclimatised at 15°C, were challenged with a $10^{-1.5}$ dilution of stock B/Lee virus. At 16, 34 and 67 hours after inoculation, three mice of each sex were sacrificed, the lungs removed, and quickly frozen individually in sterile porcelain mortars at -60°C. Five mice of each sex were kept as controls, and all the male control mice had died by the 7th day post-inoculation. One control female mouse died on the 7th day, and the remaining animals were then killed and autopsied. Male control mice had a mean M.S. 4.20 and the corresponding figure for females was 2.66.

The lungs which were collected at 16.3, 34 and 67 hours post-inoculation, were held in the porcelain mortars in an electrically operated storage cabinet until it was convenient to investigate their virus content. To carry out this investigation, two mls. of standard medium were added to each mortar, and the lungs were ground up in the frozen state. The resulting tissue suspensions were spun clear, and the supernatant titrated for infective virus. Figure 1 shows that sampling at the times stated gave no indication of major differences in the growth rates of the virus in the two sexes. There was also no evidence that the final titre of virus achieved in the lungs of the female mice was less than that in the male mice. A

barely detectable level of haemagglutinin was found in all the suspensions from mice sacrificed at 67 hours, and there was therefore no indication of the formation of exeess non-infectious virus in the male mice. The difference between the sexes appears to be due to the increased severity of the host reaction in male mice, rather than to any increase in virus proliferation.

Summarising the evidence of the influence of host factors, it is now evident that these will vary with different strains of challenge virus.

Mouse lines have been shown by others to have different reactions to influenza viruses, and the striking effect of age on the outcome of infection observed by Kalter with A/PR8 virus in Swiss mice, was not paralleled with A/Swine virus in the work just reported. Sex has been found to be important with only one strain of virus, although this factor had a like effect in two widely different lines of mice.

This phenomenon of sex differentiation has, so far, only been demonstrated with one line of the B/Lee strain. It is possible that other lines of this virus, most of which have undergone many egg and mouse passages since the virus was first isolated by Francis (1940), may not produce different mortalities and levels of lung consolidation in male and female mice. Weight was not implicated as a factor influencing the outcome of infection.

To control or eliminate host factors in influenza challenge

tests it is necessary to study each virus strain separately in the mouse lines available for testing. It may be possible to increase the sensitivity of the assay system by discovering the most sensitive mice, the more reactive sex, or by carefully choosing a suitable age for the mice. Weight may yet be found to be important, and the best way to uncover the optimal system for assay purposes is by means of a factorial experiment. Unfortunately, these are expensive, time consuming, and sometimes nullifying accidents may occur which even the most careful experimenter cannot always prevent.

C. Environmental Factors

1) Temperature and Humidity: - The effect of temperature on the outcome of infectious disease has been studied since the beginnings of microbiology. Domestic fowls do not normally suffer serious ill effects from injection with B. anthracis. A transitory pyrexia of no more than a few hours, and no trace can be found of the injected organisms although quite large doses may have been employed. Pasteur, Joubert and Chamberland (1878) reported that by immersing the birds' legs in water at 25°C subsequent to inoculation, a typical septicaemia and death resulted. Wagner (1890) found that B.anthracis does multiply readily in normal fowls, but that there is a rapid rise in the number of phagocytic cells at the site of inoculation which continues until all the bacteria are engulfed - coinciding with the end of pyrexia. In chilled birds the phagocyte response is very much reduced, and the bacteria continue to multiply freely, producing a fatal septicaemia.

Since these pioneer observations, a large number of papers have appeared dealing with the influence of environmental temperature on the outcome of experimental infections, and Sulkin (1945) has pointed out that sometimes conflicting reports have appeared with the same agent and the same animal species. The subject has been critically reviewed recently by Bennett and Nicastri (1960), and it is merely necessary to draw attention, as they do, to the lack of real evidence for many of the plausible theories advanced to explain the effect of environmental temperature. Since that review was written, Marshall (1959) has repeated and greatly extended the observations of earlier writers which showed that the outcome of myxoma infections in rabbits was largely dependent on the ambient temperature at which they were held. Mortality rates and severity of symptoms due to a slightly attenuated strain of myxoma virus were both markedly influenced, and environmental temperature had a sparing effect which was accompanied by a lessening of the maximum titre and duration of viraemia.

In the particular case of influenza, it is a commonplace observation that epidemics of human influenza tend to occur during the winter months. Andrewes (1959) discusses the implications of this "winter effect" and he regards temperature per se as likely to be unimportant because severe epidemics occur in tropical countries. Shope (1955) found that swine infested with lung worms infected with influenza virus could often be provoked into clinical influenza by exposure to adverse weather conditions. Hyde (1942) found that influenza virus grows in the rabbit lung following intranasal

inoculation, and he carried the virus through twelve passages without loss, but with only "inapparent" infection. Panthier et al. (1947) subsequently showed that clinical symptoms appear when rabbits are kept at a low environmental temperature. Saslaw et al. (1946) recorded an increased severity of symptoms in monkeys (Macaca mulatta) kept at 4° - 6°C, following intratracheal inoculation with influenza virus. Other reported experimental work has been carried out using mice, but it was of limited extent, and conflicting results were obtained. Sarracino and Soule (1941) found that following intranasal challenge with PR8 virus, there was no detectable difference in the outcome of influenza infection if the mice were transferred for various times to hot (37°C with 100% Relative Humidity) or cold (soaked in water and then 5°C) environments.

Sulkin (1945) used a high level of challenge with the same strain of virus (A/PR8), and demonstrated a highly significant difference between the mean extent of lung consolidation in mice 5-6 days after challenge, if they were held intermittently at 35°C and at 15.6°C (Relative Humidity 70% in each case). The pulmonary involvement of mice held at 21.0 - 25.5°C was intermediate in extent and probably significantly different from those held at 35°C. Other groups of mice were used to estimate the mortality by the 10th day; and here the results, although less significant, showed a similar trend viz:- a lower environmental temperature resulted in a higher percentage mortality, and reduction of the mean survival time. Re-examination of this work indicates that the significance of these

differences would be increased if mice which died on the third and fourth days were included in the results. Apparently no attempt was made to autopsy these mice to ascertain the cause of death, which was most likely to be due to influenza consolidation. Briody et al (1953) found that adaptation of an influenza A1 strain to one heterogenetic line of albine mice (N.I.H.) was greatly accelerated by holding the mice at 5°C. With an inbred strain of mice there was only slight enhancement of the adaptation process at 5°C as compared with room temperature. They found that the titre (Egg I.D. 50) reached by the unadapted virus in the lungs of the N.I.H. strain mice four days after inoculation was 100-fold greater in the animals held at 5°C than in those at room temperature.

Detailed examination of the effect of the ambient temperature on the course of influenza virus infection in mice became necessary when it was observed in a preliminary challenge experiment, that the level of virus required to produce a given degree of lung consolidation rose more than 10-fold during a heat wave which raised the temperature range of the animal room from 21 - 24°C to 29-31°C. Since the other variables had all been carefully controlled, and the infectivity of the virus strain did not change during the challenge procedure, and the mice were homogeneous as to strain, weight, sex and age, it was likely that the cause of the change was related to alteration in the physical environment in the animal room, particularly the ambient temperature.

Investigations of the Effect of Variation in the environment on influenza in mice

These experiments were carried out in insulated cold and hot rooms adjusted to the maximum and minimum temperatures stated in each test. For intermediate temperatures, an air-conditioned room in the animal house was adjusted to 22 - 23°C. Continuous records of the temperature and relative humidity (hair hygrometer) were maintained prior to and during the challenge experiments. Temperature and humidity data, which showed that the environment remained stable during the test period, related to the circulating air of the room in which the mice were held; and it was not possible to examine the micro-climate of each tin. The effective humidity may have been higher in tins because of uncontrollable factors related to the constant soiling of the sawdust bedding by the mice. It was assumed that this effect (if any) was of no significance. A number of fans were arranged in each room so that there was a constant movement of air around and into the tins, which could be checked by observing the ruffling of the fur of the mice. This had a two-fold purpose (a) minimising the possibility of variation in the micro-climate (temperature and humidity) in each tin by constant air circulation, and (b) ensuring that mice were not distressed at low temperatures by providing that they were held in constantly moving air as recommended by Briody et al. (1953) . A number of strains were tested and in each case the viruses were inoculated over a large dose range. In one case - Strain B/Lee - the effect of temperature on the outcome of infection was also tested in old mice of which there were only a limited number

TABLE III - 10

Effect of environment on B/Lee infection in mice

Average M.S. of 10 mice at each dose level for each temperature.

8 weeks old mice, 4 females and 6 males per dose.

Environment		Dilutions of challenge virus					
Temperature	Relative Humidity	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
1) 9-10°C	64%	4.25	3.35	1.86	1.07	0.74	0.52
2) 22-23°C	40%	2.95	2.10	1.78	0.67	0.69	0.20
3) 30°C	35%	2.05	1.44	0.46	0.24	0.03	0.04

Average M.S. for each sex with 10^{-2} to 10^{-4} dilutions of challenge virus.

Environment	Dilutions of challenge virus							
	10^{-2}		10^{-3}		10^{-4}		10^{-5}	
	M	F	M	F	M	F	M	F
1	3.83	2.63	2.10	1.50	1.15	0.95	1.00	0.35
2	2.20	2.00	2.13	1.25	0.87	0.38	0.78	0.55
3	1.77	0.95	0.52	0.37	0.25	0.25	0.03	0.03

TABLE III - 10a

Effect of environment on B/Lee infection in older mice

Average M.S. of 6 mice at each dose level. 6 - 7 months old mice, 3 males and 3 females per dose.

ENVIRONMENT		Dilutions of Challenge Virus				
Temperature	Relative Humidity	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
9-10°C	64%	3.00	1.63	1.20	0.88	0.50
30°C	35%	1.92	0.40	0.33	0.12	0.10

available.

Large numbers of mice of the heterogenous W.E.H. strain were acclimatized at three different temperature levels during a period of one week prior to challenge, and were then weighed. Two age levels were available, 6 - 7 months old mice, which were distributed in groups of 3 male or 3 female mice per cage, because of their size (39 - 52 g.), and 8 weeks old mice which were in groups of 4 females or 6 males per cage (22 - 30 g.) because more males than females of this age were available. The 8 weeks old mice were allocated according to sex so that there were approximately equal numbers of the same weight in each cage of males and in each cage of females. There were too few of the older mice to allow an even distribution according to body-weight; these were allocated so that the average weight of the mice in each tin was the same for each sex.

Challenge with graded doses of virus diluted in standard medium was carried out as before, and mouse scores were estimated from specific deaths and the lung lesions of the mice which survived for 7 days after inoculation.

In the first experiment B/Lee influenza virus was used to challenge mice of both ages. Table III - 10 gives details of the environments, and shows the mean M.S. for each dose level in 8 weeks old mice, and also presents the means for each sex with four of the challenge virus suspensions. Table III - 10a shows the mean M.S. for

Results of analysis of variance - Ambient Temperature with two strains of virus.

Based on data for B/Lee (Table III-10) and for A/Swine (Table III-11).

Source of Variation	Significance
Strain	***
Dose	***
Temperature	***
Strain-Dose	***
Temperature-Dose	N.S.
Strain-Temperature	N.S.

Levels of significance are denoted as in Table III - 3.

TABIE III - 11

Effect of environment with A/Swine virus in mice

Average M.S. of 10 mice at each dose level for each temperature

All 8 weeks old, 4 females, 6 males per dose/temperature.

Environment		Dilutions of challenge virus					
Temperature	Relative Humidity	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
8-10°C	64%	4.95	4.75	3.38	2.46	0.67	0.95
22-23°C	40%	4.45	3.61	2.58	1.70	1.09	0.54
30°C	35%	4.35	2.81	2.33	1.14	0.54	0.10

TABLE III - 12

Effect of environment with Strain A/PR8

Average M.S. of 7 mice at each dose level for each temperature.

10 weeks old male W.E.H. mice

Environment		Dilutions of challenge virus			
Temperature	Relative Humidity	10^{-2}	10^{-3}	10^{-4}	10^{-5}
15°C	55%	5.00	4.43	4.01	3.35
30°C	35%	4.25	3.02	2.83	1.47

Undiluted A/PR8 challenge virus contained $10^{7.45}$ LD_{50} per 0.05 ml allantois-on-shell.

six older mice at each dose level in two different environments. These results clearly demonstrate the influence of environment, with an enhanced effect of B/Lee infection in the cooler, more humid rooms. The effect was shown to occur regardless of the age or sex of the mice, although females were less affected at each dose level than males, in each environment.

A second experiment, in the same range of environments, was carried out at the same time, using A/Swine virus for challenge of 8 weeks old mice. The average M.S. for the 10 mice at each dose level are presented in Table III - 11, and these results provide further proof that the environment has a considerable effect on the outcome of influenza virus infection in W.E.H. mice.

From these experiments it is impossible to completely separate the effects of temperature and humidity. However, challenges with both B/Lee and A/Swine viruses in 8 weeks old mice at the two warmer temperatures were carried out in relative humidities which differed by only 5% in the range of moderate humidities. It is unlikely that this difference was large enough to have produced significantly different effects on the animals. Accordingly, analysis of variance was performed on the combined results of the challenges with B/Lee and A/Swine viruses at 22 - 23° and 30°C in 8 weeks old mice. Details of the individual results are given in Appendix 2, Table E, and details of the analysis are in Table F of the same Appendix. There is a highly significant difference between the results obtained at the two temperatures, and

TABLE III - 13

Effect of environment using Influenza Virus Strain A2/Singapore/ 1/57M in C57Bl mice

Average M.S. of 5 mice at each dose level for each temperature.

All males, 12 weeks old.

Environment		Dilutions of challenge virus				
Temperature	Relative Humidity	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}
12°C	55%	5.00	4.19	2.00	0.54	0.04
30°C	35%	2.50	0.08	0.02	0.00	0.00

it would appear that environmental temperature per se is a factor which may influence the outcome of influenza virus infection in mice.

The effect of environment was also tested using the A/PR8 strain for challenge in 10 weeks old male W.E.H. mice. Seven mice were used for challenge at each dose level in two environments which differed by 15°C and 20% relative humidity. Constant air circulation was maintained as before, and the mice were acclimatized at the respective temperatures for one week before intranasal inoculation. Calcium magnesium saline was used as diluent for preparation of the virus suspensions. The data for the two environments and the results of the challenge, calculated as the mean M.S. for each dose level, are shown in Table III - 12. The effect of the environment is just as evident with A/PR8 virus as with the B/Lee and A/Swine strains.

Effect of environment on influenza virus infection in C57Bl mice

C57Bl mice were used in an experiment to test the effect of environment on influenza virus challenge in a strain of mice other than the W.E.H. line. Male mice, 12 weeks old, were distributed at random in groups of five and equal numbers were acclimatized at 12° and 30°C for one week. They were then challenged with graded doses of A2/Singapore/1/57M, which is a mouse adapted line of a standard "Asian" virus. The virus dilutions were made in calcium magnesium saline. The results, which are presented in Table III - 13, show a large difference in the response of these

mice at the two temperatures. The difference is far greater than was observed with W.E.H. mice, although the A2/Singapore/1/57M strain was not tested in these random bred animals, and valid comparisons are not possible. During the course of these experiments it was observed that male C57Bl mice, if less than 9 weeks old, were liable to die within three hours of transfer from an environment with an ambient temperature of 20°C to one with an ambient temperature of 12°C. Therefore it seems likely that the extreme sensitivity to cold of the line of C57Bl mice which were used may have been the cause of the marked differences in response with the two environments.

Investigation of sparing effect of increased environmental temperature

The effects of the environment on experimental influenza in mice were further investigated to obtain a better understanding of the mechanism involved. Influenza viruses produce greater yields when cultivated in eggs at 35 - 36°C than at higher temperature, (Miller, 1944, McLean et al 1944). It was therefore possible that the reason for the lowered severity in mice at the higher temperature was the failure of the virus to reach a maximal titre. This was made to appear more likely because mice have a less effective heat control mechanism than many other animals (Mills, 1939). Numerous papers give details of the extreme variability of rectal temperature in mice with environmental temperature and humidity. Meader and Marshall (1938a and 1938b), Goldfeder (1940),

McLaren (1961) and others have studied this phenomenon. In this present work the variability was confirmed for large numbers of the stock W.E.H. mice in connection with the problem of environment on antibody production reported in Chapter IV.

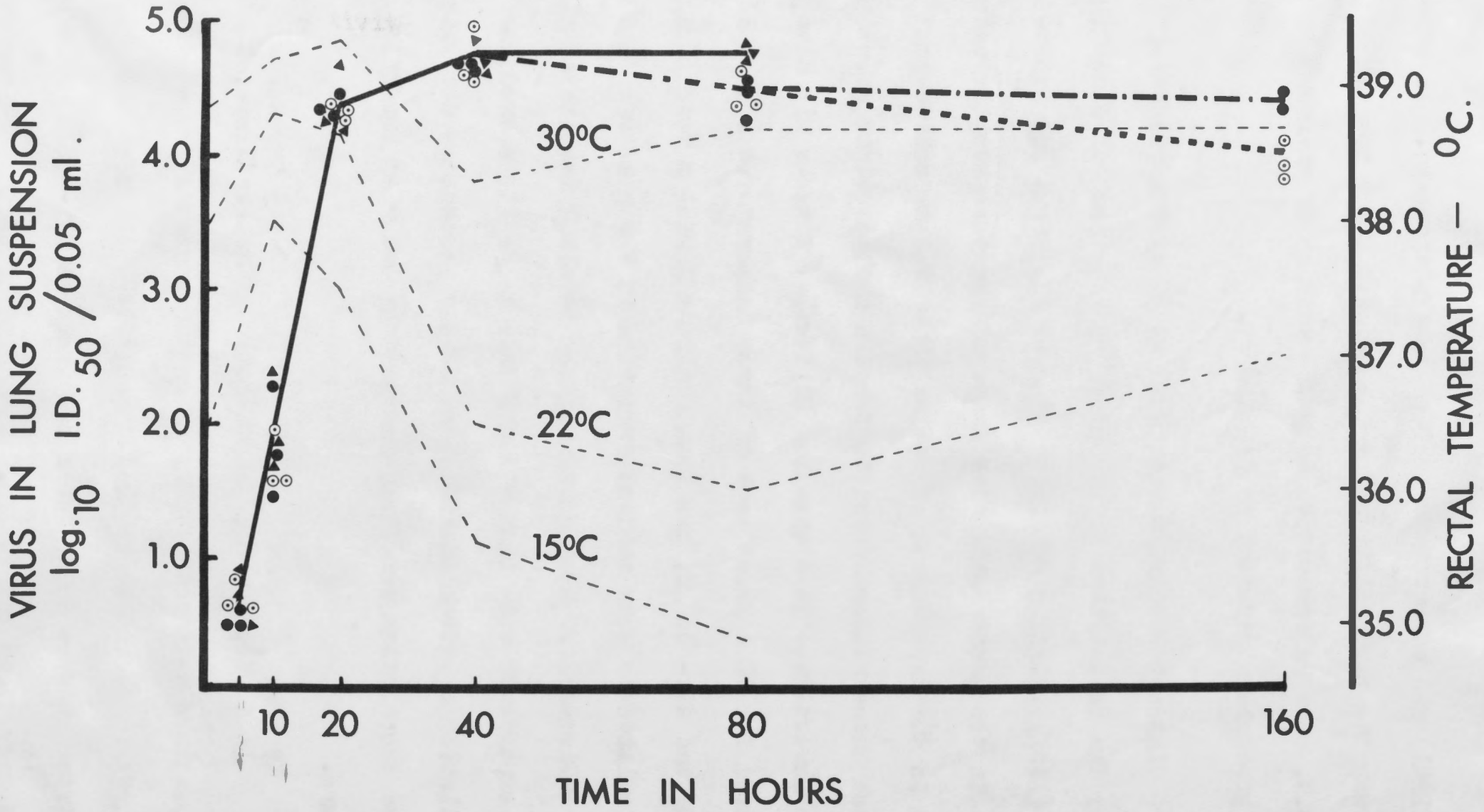
Against the hypothesis that virus growth was being limited by the temperature of the mice, was the work of Ginsberg and Horsfall (1952) relating multiplication of PR8 virus and extent of pneumonia in the mouse lung. The range of doses showing temperature variation in the experiments of Tables 10 to13 went well beyond the level which those authors found to produce maximal virus growth in mice. To investigate this question 21 female, 8 weeks old mice were acclimatized for 10 days at each of three temperatures : 15 - 16°C; 22 - 23°C; and 29 - 30° C, and then challenged with a dose of A/Swine virus calculated to show maximal variation in the mice over this range of environment. Examination of the detailed results of the previous experiment with this virus (Table III - 11) showed that with a 10^{-3} dilution of stock challenge virus the percentage mortalities 7 days after inoculation were 100,40 and 30 in order of increasing temperature.

At 5, 10, 20, 40, and 80 hours after challenge, 3 mice at each temperature were sacrificed, after their rectal temperatures had been recorded. At 160 hours post-inoculation, all mice held in the 15°C environment were dead, but 2 survivors at 22 - 23°C and 3 at 30°C were also sacrificed and tested. The lungs of each mouse were quick frozen in a porcelain mortar pre-chilled to -60°C, ground with 2 mls

FIGURE III - 2. Growth of influenza virus in the lungs of mice held in three different environments. Development of hypothermia in the course of infection.

Infectivity tests : - 30°C ⊙----⊙; 22°C ●-·-●; 15°C ▲——▲.

Mean rectal temperatures ---------- None of the mice at 15°C survived to 160 hours.



Eagle's basal medium (1955) and spun clear in a refrigerated centrifuge. The supernatant fluids were then individually titrated for virus content. The virus infectivity titrations were carried out in allantois-on-shell (Fazekas de St. Groth and White, 1958), and a preliminary survey using 10-fold dilution steps was followed by a more accurate titration using doubling dilutions. Since the allantois-on-shell titration allows eight replicates per dilution, the results were sufficiently accurate to show that there was no significant difference in the rate of virus multiplication nor in the final titre obtained in the lungs of mice held at the 3 temperatures. (See Fig. 2.). It can also be seen that the progress of infection was accompanied by a much greater drop in mean rectal temperature in the mice kept in the cooler environments.

There was no significant difference in the levels of haemagglutinin detectable in the lungs of the mice held at the different temperatures and sacrificed at 40 and 80 hours.

Interferon levels in the lung suspensions were also tested, using a modification of the method of Isaacs and Hitchcock, (1960). The centrifuged lung suspensions were, however, not dialysed against buffer at pH2.0, but sufficient 0.1N HCl. was added with rapid mixing, to lower the pH to 4.0 which is sufficient to inactivate influenza virus (Stock and Francis, 1944). After standing overnight, the samples were readjusted to 7.4 and tested for Interferon using EMC virus in mouse embryo fibroblast tissue culture. No evidence of a reduction in the number of plaques was found at the lowest dilution

TABLE III - 14

Effect of transfer from 15° to 30°C. environment after intranasal challenge.

10 weeks old female W.E.H. mice.

Post inoculation time of transfer to 30° Room Hours	Average M.S.	Day of Death
Control mice (not transferred from 15° Room)	5.00	3,4,4,4,4,4.
56	4.78	3,4,4,5,5,6.
24	4.40	4,4,5,5,5,S.
0	3.22	5,6,7,S,S,S,S,S.
Control mice acclimatized and held in 30° Room	2.64	5,7,S,S,S,S.

All challenged with $10^{-2.6}$ dilution of stock A/Swine virus.

tested (1:5), in any sample, and no comparison of interferon levels in the lungs of the mice at the three temperatures was possible.

Effect of change of environment on influenza virus infection in mice

An experiment was carried out to discover the effect of changing the ambient temperature on the outcome of influenza virus infection in W.E.H. mice. At various times after infection mice were transferred to alternative environments and the course of the disease was followed until seven days had elapsed. The experiment was then terminated with autopsy of the surviving mice and estimation of the extent of lung consolidation.

A dose level of A/Swine virus was again selected which would produce a maximal difference in response to the two environments, 15°C, with a relative humidity of 55%; and 30°C, with a relative humidity of 35%. Sufficient random bred W.E.H. mice were acclimatized in each environment and were then challenged with $10^{-2.6}$ and $10^{-3.0}$ dilutions of the stock A/Swine virus; the higher dilution being used for those animals which were acclimatized to the higher temperature. At selected times after challenge, groups of the animals were transferred to the alternative environment, and the outcome of infection was recorded as in previous experiments. Table III - 14, which refers to mice challenged at the $10^{-2.6}$ level, shows that mice were spared if they were transferred from a cold environment immediately after inoculation, and there were no deaths in this group until after all the control mice in the 15° room died.

TABLE III - 15

Effect of transfer from 30° to 15°C. environment after intranasal challenge

10 weeks old female W.E.H. mice.

Post-inoculation time of transfer to 15° Room Hours	Average M.S.	Day of Death
Control mice (not transferred from 30° Room)	2.47	6,6,S,S,S,S.
120	4.50	6,6,6,6,6,6,
90	4.67	4,4,5,5,6,7.
56	4.50	5,5,6,6,6,6.
24	4.75	4,4,4,5,5,6.
0	4.83	3,3,4,4,5,5.
Control mice acclimatized and held in 15° Room	4.58	3,4,5,5,6,7.

All challenged with 10^{-3} dilution of stock A/Swine virus.

Transfer to 30° at 24 hours also delayed the time of death, and one mouse survived until the termination of the experiment, although the extent of lung damage at autopsy was considerable (M.S. 3.0). The sparing effect was not apparent in mice transferred after 56 hours, except that there was an indication that time of death was delayed.

Mice acclimatized at 30° and transferred to 15° immediately after inoculation, with a 10^{-3} dilution of virus were visibly more affected 48 hours later than controls which were maintained at 15° prior to and during the experiment. Table III -15 shows that "chilling" at the time of infection reduced the mean survival time from 5 to 4 days. Transfer from the warm environment at later times resulted inevitably in death of the mice, but there was a progressive increase in survival time with increasing delay of the transfer. No experiments were carried out to discover whether "chilling" produced a sudden increase in virus or merely precipitated a violent host reaction to the already existing infection.

It is quite apparent that for quantitative studies of influenza virus infection in mice, a stable environment must be provided for the animals on test, and there is also a need for acclimatization to the test environment before commencing experiments of this nature.

Cross Infection

Eaton (1940) made the first observation of cross infection by influenza virus strains with subsequent demonstration of lung lesions and occasional deaths in contact mice. His work was carried

out using commercial Swiss mice, presumably not inbred, and he found a variation in the "infectivity" of different virus strains. Kilbourne (1960) reported that he had often successfully repeated these experiments, but Andrewes stated in the same discussion that he had been unable to repeat this work although he had made repeated attempts using Eaton's strains of virus and mice.

The following experiment was designed to disclose the effects of variations in strains of mice, and of environmental temperature, on the possibility of cross infection in experimental influenza in mice. Female mice were distributed in tins so that for each strain there were equal numbers of the same weight, six mice per tin. After 4 days acclimatization, female mice of the same breed, similarly acclimatized, were infected with A/Swine virus, the least infective strain in Eaton's (1940) work, and these were added 2 hours later, 2 to each tin. Dose levels were chosen so that the lower dilution should cause 100% deaths within 7 days in the "inoculated" mice, regardless of environment, in each strain of animal. In the actual experiment, all the "added" mice died within the period of test except for one W.E.H. mouse which was in the warmer environment, and which showed 75% lung consolidation at autopsy. On the 8th day after introduction of the infected mice, all the test mice were sacrificed, the extent of lung lesions was estimated, and the lungs were frozen in porcelain mortars at -60°C and ground up with 2.0 ml. of standard medium. After centrifugation of these lung suspensions, the supernatant fluids were examined for the presence of virus by inoculation into the allantoic cavity of 11 days old embryonated eggs,

TABLE III - 16

Cross Infection in Experimental Influenza

Outcome of introducing 2 infected mice into tins of 6 mice of the same strain.

Mouse Strain	Environment		2 mice inoculated with A/Swine virus		Test Mice	
	Temperature	Relative Humidity	Challenge Dilution	Mortality	Lung lesions at 7 days	Virus in lungs
C57Bl	15°C	65%	10^{-3}	2/2	3.0, 1.0, 1.0, 0.2, 0.0, 0.0	6/6
	30°C	35%	10^{-3}	2/2	All -ve	1/6
W.E.H.	15°C	65%	10^{-2}	2/2	All -ve	0/6
	15°C	65%	10^{-3}	2/2	All -ve	0/6
	30°C	35%	10^{-3}	1/2	All -ve	0/6

and subsequent testing of the allantoic fluid for haemagglutinin after 48 hours incubation. Results are presented in Table III -16, and it is obvious that challenged C57Bl mice may experience cross infection if they are held in a cool environment. Virus was also found in the lungs of one of these mice which had been kept at 30°C. W.E.H. mice showed no evidence of cross infection, and the genetic background of the mice appears to be an important factor in determining the occurrence of this phenomenon.

The possibility of cross infection, with its implication of multiple challenge, must be considered and prevented, otherwise the use of susceptible strains of mice for experiments to assay immunogenicity of vaccines is precluded. As a model subject for use in the practical study of overall effectiveness of vaccines, however, susceptible mice may well be preferable, since it is reasonable to assume that the population of humans at risk in any epidemic, is subjected to, and is susceptible to, multiple exposures.

2) Barometric Pressure: - The possibility of "atmospheric pressure" influencing the outcome of infection has been referred to in the discussion of the effect of varying oxygen tension. The subject is worthy of investigation, but could not be attempted in this study because of time limitations. It may be assumed that the precaution of housing all animals under test in the same room will prevent any possible barometric pressure effect from introducing bias to assay results.

In summary, environmental temperature has been shown to

be a major factor in deciding the outcome of influenza virus infection in the laboratory mouse. The sparing effect of increased temperature was not accompanied by a reduction in the rate or extent of virus proliferation, and was not demonstrable if the test animals were transferred from a cold environment after maximum virus growth had been achieved. After infection in a warm environment, transfer to a cool temperature at any time up to 5 days post-inoculation, produced a rapid increase in the severity of the disease. In a cool environment, some strains of mice may be liable to multiple infection, which, unless prevented, would preclude their use in assay studies, although this characteristic may be of value in other directions.

The results obtained in these experiments lead to the important conclusion that any assay system for influenza virus, and therefore for influenza vaccine immunogenicity in mice, must be carried out under carefully controlled conditions of environmental temperature, and probably humidity. In this present study it was not possible to analyse the effect of the latter, but it has been shown that it is possible to vary the rectal temperatures of mice by raising the humidity (Marshall and Meader,1938b), and it may be inferred that the outcome of influenza virus infection in mice would be correspondingly affected. The ultimate proof awaits further experimental work. For all challenge tests described in this thesis the factor of humidity was carefully controlled.

Barometric pressure has not been investigated as a factor which might affect the result of influenza infection in mice.

D. Other Factors

Other factors which have been studied in influenza virus infections in mice e.g. fatigue, by Sarracine and Soule (1941), were not considered since there is no reason to suppose that they will be of importance in a properly conducted study. A similar observation can be made about unhealthy or pregnant animals which must be rejected if they appear amongst test mice. Indeed, if unhealthy mice appear in the stock of mice for test, it is probably wiser to obtain a fresh supply of animals, than to attempt to use survivors.

DISCUSSION

The most important conclusion to be drawn from this work is that the factors influencing the response of mice to influenza infection may vary with different strains of the virus, and with the genetic background of the mouse line which is used. In the design of a test for immunogenicity, this introduces a difficulty which is not overcome by simply using equal numbers of mice of both sexes, or of different ages, or of corresponding weights, at each dose level, for all virus strains. This would merely increase the variance and tend to "flatten" the response curve, reducing the sensitivity of the test. For a maximum sensitivity it is necessary to examine each host factor in detail for each strain of virus and mouse, preferably using a factorial experiment which will disclose interactions. Table III - 8 shows that the slope of the dose response curve to B/Lee virus in female C57Bl mice is much flatter than for male mice. This indicates that only male mice should be used, if C57Bl mice are the

available test animal for this virus. Table III - 7, however, would indicate that there are roughly parallel slopes for both sexes in W.E.H. mice, and the use of either but not both sexes of this strain would be permissible.

The demonstration that variation of the environment has a major effect on the outcome of influenza infection, indicates that an animal room with areas of uneven temperature or humidity is unsatisfactory for accurate assays. It does not suffice to distribute mouse containers in such a way that equal numbers at each dose level will be in the same part of the room. All mice must be subjected to the same external conditions, and this can only be achieved by using an efficient air circulation system and maintaining a constant temperature and humidity. A factor of equal importance is the proper design of the containers for the animals; these must permit ready air circulation, and the food hoppers and water bottles should allow the animals ready access to food and drink at all times.

The finding that age of mice did not influence the outcome of influenza virus infection was at variance with the observations of Kalter (1949) who studied challenge of commercially bred Swiss mice with A/PR8 virus. In the present investigation, A/Swine virus was tested in a random bred line of mice, and evidence was also presented that B/Lee virus produced comparable mortalities and levels of lung consolidation in two widely spaced age groups of the same animals (Tables III - 10 and 10a). Similar observations were also made with A/Swine and B/Lee viruses in small groups of C57Bl mice ranging in

age from 10 to 25 weeks. It is most probable that the variation in response according to age which was found by Kalter was a characteristic of the strain of mice which he used.

Two interesting, and at present unexplainable phenomena have been disclosed during these investigations. These are the different levels of severity of reaction between male and female mice to infection with a particular strain of influenza virus, and the sparing effect of warm environments despite maximal growth of the virus in the lungs of the mice. The increased severity of the outcome of B/Lee infection in male mice may be merely characteristic of the particular passage line which was used. The virus was originally isolated by Francis (1940) from a mild epidemic of influenza in a hospital for children with rheumatic conditions (Reyersbach et al, 1941). The incidence of infection in this closed community was higher in girls than in boys although the difference is not particularly significant. There was also no indication of increased severity in boys.

An unexpected finding was that the severity of the outcome of infection was not related to the level of virus multiplication in the mouse lungs. This does not contradict the findings of Ginsberg and Horsfall (1952), since the level of virus challenge employed was above the range which they studied. It is also possible that an investigation using smaller doses of challenge virus would disclose differences in the rate of virus proliferation due to environmental factors, or, in the case of the B/Lee virus

used here, to the sex of the mice.

In an experiment designed to study the possible causes of the sparing effect of a warm environment no interferon could be detected in the lungs of influenza virus infected mice. There was insufficient material to test for interferon at the level used by Isaacs and Hitchcock (1:2); and the higher dilution may have prevented its detection. Alternatively, since the processes of acidification and neutralization both resulted in heavy precipitates which were spun down and discarded, it is conceivable that any interferon present may have adsorbed to one or other of these precipitates, and been lost. However, the infectivity of the virus used for mouse challenge in this experiment was much higher than that used by Isaacs and Hitchcock, and it seems likely that this provides the explanation for inability to detect interferon. The size of the challenge dose in the mice was such that it may have overwhelmed the cells to such an extent that the mechanisms involved in the production of interferon were not able to function. In any case it is unlikely that interferon as such is concerned in the particular environmental phenomenon described in this section; this is clear from the failure of increased temperature to influence virus multiplication, although its sparing effect on virus pathogenesis is highly significant. The answer will have to await further experimentation, but it is noteworthy that Baron and Isaacs (1962) were unable to detect interferon in the lungs of fatal human cases, where, presumably, maximum virus proliferation would also have occurred.

Another finding of interest in the study of environmental effects on influenza virus infected mice was that during the course of the disease there was a greater drop in rectal temperature in animals which were held at lower temperatures. Since the outcome of infection was also more severe in these mice, this observation was in keeping with that of Yang and Evans (1961), who showed that the time of occurrence and level of hypothermia which followed intranasal A/PR8 inoculation in mice kept at 24 - 27°C was related to the maximum extent of pulmonary lesions rather than the maximum virus titre in the lungs.

The increased severity noted when mice were transferred from a warm to a cold environment may indicate that the "winter factor" of Andrewes (1959) is in fact a "chilling factor", which is far more likely to be of importance in winter than summer months. Warburton and Duxbury (1959) reported on an outbreak of influenza due to A2 virus in an isolated community in Northern Territory of Australia in which a "chilling factor" appeared to be present. The population of slightly more than 300 persons, which had not been affected by the 1957 epidemic of A2 virus influenza, experienced a sharp and widespread outbreak of clinical influenza following a sudden drop in the environmental temperature. There was clear evidence that the virus had been "seeded" into the community 2 to 3 weeks previously, but no cases of influenza occured until there was a change in the weather.

With regard to the demonstration of an increase in

cross infections with C57 Bl mice if they were held in a particular environment, this may well have been as much conditioned by the effect of the relative humidity on the virus as by the effect of the environment on the animals. Lester (1948) showed that the infectivity for young white mice of atmospheres containing atomised A/PR8 influenza virus was a function of the relative humidity of the atmosphere when the mice were held in a 22 - 24 °C environment. His results show a definite minimum infectivity at a relative humidity of 45%, with peaks at the extremes of dryness and saturation. It is possible that other results might have been found with other ambient temperatures, but relative humidity appears to be an important factor influencing virus communicability in mice.

SUMMARY

1) Body-weight, sex and age of mice, from 10 to 22 weeks old, did not influence the outcome of infection with A/Swine virus in a random bred line of mice.

2) Male mice, of both random bred and inbred lines of mice, were found to be more susceptible than females to intranasal infection with a mouse adapted line of B/Lee influenza virus. The increased susceptibility was not accompanied by an increase in the extent of virus proliferation in the lungs of the mice.

3) Environmental temperature had a major effect on the response of both random bred and inbred lines of mice to infection with a number of strains of influenza virus.

4) This effect was not due to a reduction in the rate, or the extent, of virus proliferation in the lungs.

5) After infection with a large inoculum, transfer from a warm to a cold environment precipitated a fatal reaction in mice.

6) Transfer from a cold to a warm environment had a sparing effect only during the early stages of the infection.

7) Cross-infection occured with an inbred line of mice when these animals were maintained in a cold environment.

CHAPTER IV

THE ANTIBODY RESPONSE OF MICE TO INFLUENZA VIRUS VACCINES

Fazekas de St. Groth, Donnelley, and Graham (1950, 1951, 1954) elucidated the role which specific antibody plays in immunity to influenza virus infection in mice. The protective function of antibody was shown to be effected only by that fraction which is present on the surface of the respiratory tract, and which presumably neutralizes virus before it can initiate infection. Individual variation in the distribution of antibody between the circulation and the respiratory tract was implicated as one of the reasons for the lack of exact correlation between antibody level in serum and immunity as assessed by direct challenge. The level of antibody which appeared in the serum as a result of vaccination was, however, shown to have a major influence on the amount which was present in the bronchi (Fazekas de St. Groth and Donnelley, 1951a). Consequently, before proceeding to a study of direct challenge tests in vaccinated mice, some of the factors which might influence the serum antibody response, apart from dose, were examined.

Methods of Titration

For the work reported in this chapter, 2 methods of antibody titration were employed. Neutralization of infectivity was estimated by allantois-on-shell (Fazekas de St. Groth et al. 1958), and antihaemagglutinin levels were measured by a method which is also described in that paper, after the sera had been treated with Receptor Destroying Enzyme to destroy non-specific inhibitors. The erythrocytes used in the anti-haemagglutinin tests were taken from two birds which had been selected from approximately 400 fowls;

agglutination of their red cells by B/Lee virus, previously heated to 55°C for 30 minutes, was shown to be insensitive to non-specific inhibition by normal mouse or rabbit serum which had been diluted to 1/50 in calcium saline and boiled for ten minutes. This treatment of serum does not destroy Francis inhibitor, but actually increases it 10-fold, (McCrea, 1948). Both methods of titrating influenza virus antibodies are described in Appendix 1.

A difficulty arose with the titration of mouse sera for anti-haemagglutinin in that sera from both C57Bl and W.E.H. mice were found to have high levels of natural antibody against the erythrocytes of fowls. The same result was found with 25 white leghorn and crossbred birds although the titres varied slightly with individual fowls. Time did not permit a lengthy investigation, and there is no knowledge of the application of this phenomenon to other mouse lines or to all varieties of Gallus domesticus.

Within one group of a hundred animals including both C57Bl and W.E.H. strains, the titres of natural antibodies ranged from 1/20 to 1/200 with a mean of 1/67. Therefore, for all serological work in which fowls' cells were to be used, at least 2 absorptions were performed on the lowest serum dilution, using volumes of packed fowl cells equal to the amount of serum present. This eliminated the problem of the natural antibodies, and titres as low as 1/10 could be measured.

These serological techniques were then employed to study the factors which might possibly influence the antibody response of mice to intraperitoneal inoculation with graded doses of influenza virus vaccines.

Factors influencing the antibody response to influenza virus vaccines in mice

Although the scope of this investigation was very limited, it will be convenient to adopt the classification used in Chapter III for its presentation.

A. Metabolic Factors

1) Nutritional Status: - Cannon et al. (1943) found that dietary deficiency in rabbits led to a decreased capacity to produce agglutinins. Wissler et al. (1946) showed that a similarly induced lowering of the antibody response in rats was reversible on restoration to a diet containing high quality protein. From this and later observations of a similar nature, it must be considered that nutritional status may affect antibody production in any system, including influenza virus vaccines in mice, unless the contrary is clearly demonstrated. The effect of dietary deficiency was not investigated in the present study, but a constant check was made to ensure that excess food was always available to the mice, both prior to and during immunization.

2) Water: - An experiment to demonstrate the effect of dehydration on the growth and antibody response of W.E.H. mice has already been described in Chapter III, and the results, shown in Table 2 of that

chapter, indicated that moderate dehydration for a limited time did not reduce the anti-haemagglutinin antibody response of W.E.H. mice. Nevertheless, in view of the considerable influence of this factor on food consumption in mice, it is possible that prolonged restriction of the water supply might result in a lowering of antibody titres, and frequent checking of the free availability of water should be taken as a precautionary measure.

B. Host Factors

The antibody forming capacity of animals has been shown to be influenced by their genetic background, their age and sex (Boyd, 1956). So far as age is concerned, the evidence indicates that very young and very old animals are deficient in antibody forming power, and should be avoided in experimental work on antibody production. Only sexually mature animals, at least 6 weeks old, and no animals older than 7 months, were used in the studies reported in this thesis. It was essential, however, to investigate the adequacy of the antibody responses to influenza virus vaccines in the strains of mice which were to be used, and to compare the antibody responses of the sexes in mice of the same age.

Strain of Mouse: Male C57Bl mice were obtained after weaning, 4 weeks after birth, and were held until they were 12 weeks of age. An equal number of male W.E.H. were similarly obtained so that they reached the age of 6 weeks at the same time as the C57Bl mice were 12 weeks old. In this way it was possible to compare the 2 strains

TABLE IV - 1

Average neutralizing antibody responses in groups of five mice

Geometric mean reciprocal titres expressed in $\log_{10}$ units.

B/Lee Vaccine

Strain of Mouse	Tested at (days)	Dose of B/Lee Vaccine $\log_{10}$ Haemagglutinin units 1.0	1.5	2.0	2.5	3.0	Overall Means
C57Bl	17	2.18	2.36	2.68	2.98	3.08	2.66
	29	2.24	2.42	2.48	3.08	3.28	2.70
W.E.H.	17	2.22	2.32	2.46	3.08	3.20	2.66
	29	2.38	2.46	2.44	3.14	3.22	2.73

Results of analysis of variance:- Strain of mouse and time of serum sampling . B/Lee vaccine

Source of Variation	Significance
Strain	N.S.
Time	N.S.
Dose	***
Strain-Time	N.S.
Strain-Dose	N.S.
Time-Dose	N.S.

Levels of significance denoted as in Table III - 3

in animals of essentially the same weight. Groups of five animals of each strain were injected intraperitoneally with doses of non-inactivated B/Lee vaccines ranging from 1.0 to 1000 haemagglutinin units in 3.16-fold increments. Dilutions were made in calcium magnesium saline, and the dose of vaccine was contained in 0.25 ml. which was the volume injected into each mouse. After completion of the mouse inoculations, each dilution of vaccine was titrated to ensure that there had been no loss of haemagglutinin titre during the injection operations. Twelve days later a further series were injected with the same schedule of doses, and all mice were bled 17 days after the latter injection. The individual sera were inactivated, and titrated for neutralizing antibody using the egg bit method.

Table IV - 1 shows the geometric means of the responses of both strains of mice at each dose level, tested 17 days and 29 days after inoculation. The individual results and an analysis of variance are presented in Table G of Appendix 2. This analysis indicated that there was no significant difference between the responses of the mouse strains.

A further series of mice were inoculated with non-inactivated A/Swine vaccine with doses ranging from 1 to 100 haemagglutinin units, with the same increments (3.16-fold). Ten male and ten female animals of each strain were injected intraperitoneally with each dose, and bleeding schedules were arranged so that individual sera were collected from 5 male and 5 female mice on each

TABLE IV - 2

Average neutralizing antibody responses in groups of ten mice

Geometric mean reciprocal titres expressed in $\log_{10}$ units.

A/Swine Vaccine

Strain of Mouse	Tested at (days)	Dose of A/Swine vaccine log.10 Haemagglutinin units 0.0	0.5	1.0	1.5	2.0	Overall Means
C57Bl	17	1.12	1.47	1.68	1.83	2.29	1.68
	29	1.39	1.75	2.14	2.20	2.43	1.98
W.E.H.	17	1.14	1.35	1.73	1.97	2.30	1.70
	29	1.44	1.65	2.07	2.20	2.55	1.96

Results of analysis of variance: - Strain of mouse and time of serum sampling. A/Swine vaccine

Source of Variation	Significance
Strain	N.S.
Time	***
Dose	***
Strain-Time	N.S.
Strain-Dose	N.S.
Time-Dose	N.S.

Levels of significance denoted as in Table III - 3

dose on the 17th and 29th days after immunization. Neutralizing antibody titrations were again carried out, and the results expressed as geometric means of the reciprocal titres at each dose level are shown in Table 1V - 2. The individual data for all mice, and analysis of variance of the results are given in Tables H and I of Appendix 2. No attempt has been made to analyse differences due to sex, because some of the female C57Bl mice had produced litters, whilst others were still virgin, and these mice could not be regarded as a homogeneous group. However there were no significant differences in the overall responses of male and female mice of each strain.

This experiment gave no indication of different antibody responses of the two breeds of mice with A/Swine vaccine, and it was concluded that antibody response would not be a factor influencing the outcome of challenge tests in which both strains of mice might be used. On the other hand, there was a highly significant difference between the antibody levels in both lines at the 17th and 29th days after inoculation, the 17th being sifnificantly lower than the 29th. Accordingly, in all subsequent tests, the blood sampling and challenge operations were each always completed within 20 hours, and, where possible, these were performed on the 19th and 21st days post-inoculation, respectively.

Sex of Mouse : - The anti-haemagglutinin antibody responses of males and females to influenza virus vaccine were investigated using 22

weeks old C57Bl mice. A suspension of A2/Singapore/1/57M virus was prepared and inactivated by means of minimal irradiation with ultra-violet light (see Appendix 1). Two dilutions of the vaccine, containing 400 and 800 haemagglutinin units, were made in calcium magnesium saline, and were each injected intraperitoneally into a group of 20 males weighing between 23 and 26 g. and a group of 20 females weighing between 19 and 21 g. On the 19th day post-inoculation all mice were bled, and anti-haemagglutinin titrations were carried out after the sera had been absorbed twice with fowl erythrocytes, and treated with Receptor Destroying Enzyme to destroy non-specific inhibitors. Data for individual sera, and analysis of variance, are given in Table J of Appendix 2. The overall mean antibody responses, expressed as $\log_{10}$ reciprocal anti-haemagglutinin titres, were 2.07 for males, and 2.01 for females. The difference between the anti-haemagglutinin responses of the sexes to intraperitoneal inoculation with A2/Singapore/1/57M vaccine was clearly insignificant.

The anti-haemagglutinin antibody responses of 20 male and female C57Bl mice to a single dose level, 200 haemagglutinin units, of a B/Lee vaccine, inactivated by ultra-violet irradiation, were also measured at 19 days post-inoculation. The means of the reciprocal anti-haemagglutinin titres, expressed as $\log_{10}$ units, were 1.59 for males and 1.51 for females, which were not significantly different. Despite the small number of animals tested, failure to demonstrate a significant difference between the anti-haemagglutinin responses of

the sexes indicated that sex would not be a major factor influencing the host antibody response to B/Lee vaccines

C. Environmental Factors

Temperature and Humidity: - Ipsen (1952) investigated the immune response to tetanus toxoid of mice held at 6°, 25°, and 35°C. To measure protection he used direct challenge with toxin, after all mice had been transferred to 25°C. He found a positive correlation between environmental temperature and development of resistance to challenge. Kopeloff and Stanton (1942) showed that the haemolysin response in rats to single intravenous injections of sheep red cells was reduced if the internal temperature of the animals was artificially lowered by 4° to 5°C, and 36 hours after restoration to normal temperature the haemolysin titres were found to have risen to the same level as in rats which had not been chilled. More recently Chaffee and Martin (1962) could detect no significant difference in the levels of Coliphage inactivating antibodies produced at 6 or 10 days in cold acclimatized (4°C) Swiss mice, and others held at room temperature. Their animals were acclimatized for long periods, 8 - 12 weeks, before inoculation.

The effect of environment on the antibody response of mice to an influenza virus vaccine was examined in the following experiment : -Forty male and 10 female W.E.H. mice were acclimatized for one week at 10 °C, and equal numbers of identical weight and sex were held at 30°C. The animals were segragated into lots of

TABLE IV - 3

Antibody responses to B/Lee virus at 10°C and 30°C in 10 weeks old male and female W.E.H. mice

Geometric mean reciprocal anti-haemagglutinin titres of 5 mice with identical weights

Weight (g.)	Sex	Environment	
		30°C: 34% Relative Humidity	10°C: 65% Relative Humidity
35	Male	17.4	13.9
33		24.2	22.0
33		24.9	16.2
32		34.2	21.4
31		25.2	23.9
31		25.9	26.3
23 - 24		21.1	16.4
21 - 22		22.9	24.2
30 - 31	Female	22.9	16.9
20 - 21		31.9	23.2
Overall Means		25.06	20.44

t = 2.26, and is significant at the 5% level with 18 degrees of freedom.

five of the same weight, and were all inoculated with 0.25 ml. of B/Lee vaccine containing 50 haemagglutinin units. Fourteen days later 0.5 ml. blood was taken from each mouse, and the serum titrated for anti-haemagglutinin. Table IV - 3 shows the geometric mean titre of each corresponding group of five mice, and also the overall means for each environment.

It may be seen that there is a small but definite trend towards higher levels of antibody in mice held in the warmer environment, the difference being statistically significant at the 5% level.

Discussion

The previous finding that anti-haemagglutinin antibody production was not impaired, during a period of limited access to water, was not unexpected. The most noteworthy effect of chronic dehydration is a concomitant reduction in the intake of food, and this, rather than limitation of water per se, was regarded as the factor most likely to influence the antibody response. Wilson and Miles (1955), reviewing the published reports of the effects of nutritional deficiencies on antibody production, concluded that positive effects may appear only after long periods on a depleted diet.

Despite this lack of effect on the antibody response, however, it was shown in Chapter III that restriction of access to water may influence the outcome of challenge with influenza viruses in

immunized mice and care was taken to see that water was available to the mice at all times.

The highly significant difference between the neutralizing antibody levels of both strains of mice on the 17th and 29th days after inoculation with A/Swine vaccine was due to a rise in titre during the intervening 12 days. Although there was a similar trend with B/Lee vaccine, the difference in the results on the two days was not significant.

Fazekas de St. Groth and Donnelley (1950a) inoculated W.E.H. mice with a range of doses of A/Mel vaccine by the intraperitoneal route, and studied the anti-haemagglutinin response for three weeks. Maximum titres were always reached by the 13th to 14th days, and the antibody level subsequently fell slowly to the 21st day. It is possible that the antibody response of mice to influenza virus vaccines may not follow the same course with different strains of virus.

No significant differences between the antibody responses of male and female mice at the 19th day post-inoculation were found with vaccines prepared from three strains of influenza virus. It was therefore concluded that both sexes might be used indiscriminately in experimental work on antibody production with these strains, and that the outcome of challenge tests with these viruses in immunized mice would not be influenced by differing antibody responses in males and females.

Environment was found to be a factor which might influence the antibody response of mice to a B/Lee influenza virus vaccine. However, the comparison was made between mice held at two widely spaced temperatures; fluctuations of this order may be expected to occur in the ambient temperatures of some animal holding quarters, but usually the range of temperatures would be much less, and would be unlikely to influence appreciably the antibody responses of mice to intraperitoneal inoculation with influenza virus vaccines.

Summary

1) Restriction of the water supply to mice did not affect the antibody response to intraperitoneal inoculation with an A/Swine vaccine.

2) The neutralizing antibody response of C57Bl and W.E.H. mice to A/Swine and B/Lee vaccines were essentially similar.

3) In both lines of mice there were significant increases in the neutralizing antibody titres between 17 and 29 days after immunization with A/Swine vaccine.

4) Environmental temperature had small but definite influence on the anti-haemagglutinin antibody response to B/Lee vaccine, but fluctuations in temperature usually found in holding quarters for experimental animals are unlikely to influence significantly the antibody responses of mice to influenza virus vaccines.

CHAPTER V

CHALLENGE TESTS IN IMMUNIZED MICE

The experimental work described in earlier chapters clearly showed that there were several important factors which had to be considered, and their effects minimized or controlled, in the design of mouse challenge tests for the study of influenza vaccine immunogenicity. Before attempting to set up these tests, it was decided to continue investigations into the possible influence of sex, age and body weight of mice, using challenge of immunized mice. The two following factorial experiments were planned to uncover any effects of these variables, and their possible interactions.

Effect of sex, age and body weight of animals on the outcome of challenge in immunized mice.

In the first experiment, four weeks old W.E.H. mice, approximately equal numbers of each sex, were obtained at intervals of 3 weeks, until 3 ages had been collected. When the youngest group were 6 weeks old, all mice were weighed, numbered, identified, and allocated randomly to containers according to age and sex, using the methods outlined in Chapter III. Each cage contained 6 mice.

A vaccine was prepared with the type A strain "Bel", using a single cycle of adsorption to and elution from chicken erythrocytes, and two cycles of differential centrifugation. The vaccine was not inactivated, but 0.08% sodium azide was added as a preservative, and it was stored at $4^{\circ}C$ before use.

Graded dilutions of this vaccine were prepared, in two-fold steps, in calcium magnesium saline, and 0.25 ml. of the dilutions,

TABLE V - 1

Challenge of Immunized Mice - A/Bel vaccine

Arithmetic mean M.S. for six W.E.H. mice challenged with 100 M.S. 2.5 of A/Bel virus, 21 days after immunization.

Dose of Vaccine, Haemagglutinin Units	Males: Age in weeks at immunization 12	9	6	Females: Age in weeks at immunization 12	9	6
2048	0.04	0.20	0.00	0.00	0.00	0.72
1024	0.20	0.14	0.23	0.05	0.00	0.37
512	0.47	0.42	0.62	0.12	0.10	0.75
256	1.55	1.30	0.98	0.53	1.30	1.43
128	1.20	1.25	2.25	0.87	1.83	0.82
64	1.02	1.93	1.28	1.42	1.53	1.20
32	0.95	1.53	2.43	1.65	2.78	2.53
16	2.17	1.67	1.85	2.32	3.40	2.30

Results of Analysis of Covariance - Age and Sex with Weight as co-factor

Source of Variation	Significance
Sex	*
Age	*
Dose	***
Age-Sex	*
Age-Dose	N.S.
Sex-Dose	N.S.
Weight	***

containing from 16 to 2048 pattern units of haemagglutinin, were inoculated by the intraperitoneal route into 6 mice of each age-sex group. Nineteen days after vaccination all mice were bled by the retro-orbital technique; the sera were separated and stored at -20°C.

Duplicate preliminary titrations were performed to establish the level of A/Bel stock challenge virus (see Appendix 1) which produced an average M.S. 2.5 in 12 weeks old W.E.H. mice. On the 21st day after immunization of the mice a dose of virus calculated to contain a 100-fold concentration of this level (100 M.S. 2.5) was used to challenge all mice. Infectivity titrations in surviving allantois-on-shell, and control titrations in unimmunized mice, were performed on the challenge virus before and after the animal inoculations, and at a time when approximately 50% of the operation had been completed. There was no significant difference in the virus titres, by either method, at the three sampling times. Specific deaths from influenza were recorded from the 48th hour post inoculation, and all surviving mice were autopsied after 7 days. The arithmetic means of the M.S. for each dose of vaccine in each age-sex combination are shown in Table V - 1.

Analysis of co-variance, testing for the significance and eliminating the interaction effect of pre-immunization weight, was carried out as described in Chapter III. Mice were once more allocated to "classes" based on the mean and standard deviation of the normal distribution of their body-weights. Numbers 1 to 5 were again arbitrarily allotted to classes with increasing weight for the

purpose of the statistical analysis. Individual M.S. and weight numbers are given in Appendix 2, Table K, and the analysis is presented in Table L of the same Appendix. The analysis shows that there was a highly significant negative correlation between the outcome of challenge and body-weight of the mice, and indications that age and sex of the mice might have influenced the results.

A further factorial experiment was performed in W.E.H. mice, using an A/Swine vaccine inactivated by ultra-violet irradiation (see Appendix 1) for the immunization, over a range of doses extending from 0.03 to 3000 haemagglutinin units. Serial 10-fold dilutions of vaccine were given by the intraperitoneal route to weighed, numbered, identified, and randomly distributed mice. The six dose levels were given to five mice of each sex, at each of the ages 6, 9, 12 and 15 weeks. Blood sampling was performed on the 19th day post-inoculation.

The challenge of these mice was carried out immediately after the conclusion of the challenge tests with A/Swine virus in unvaccinated mice described in Chapter III. This challenge in unimmunized mice was timed to be a preliminary titration for each age and sex of mice so that levels of virus capable of producing an average M.S. 2.5 could be calculated, and suitable challenge doses given to the immunized mice. As reported in Chapter III, no evidence of a significant age or sex effect was found, so all immunized mice were challenged with the one dilution of stock virus (10^{-3}). This was 100-fold more concentrated than the level which

TABLE V - 2

Challenge of Immunized Mice - A/Swine Virus

Arithmetic mean M.S. for five W.E.H. mice challenged with 100 M.S. 2.5 of A/Swine virus 21 days after immunization.

Dose of Vaccine Haemagglutinin Units	Males				Females			
	Age in weeks at immunization				Age in weeks at immunization			
	15	12	9	6	15	12	9	6
3000	0.00	0.00	0.06	0.12	0.00	0.00	0.26	0.06
300	0.04	0.325	0.25	0.20	0.10	0.68	0.04	0.14
30.0	0.46	0.92	1.36	1.78	0.86	0.82	1.42	1.96
3.0	1.80	1.82	1.80	3.26	2.46	1.68	3.50	2.80
0.3	1.70	1.72	2.00	2.92	3.10	3.76	2.70	2.90
.03	2.30	2.20	2.575	2.38	3.00	3.10	3.22	2.50
∅	2.20	2.06	2.66	2.46	2.40	2.30	3.04	2.80

∅ Control mice, not immunized.

Results of Analysis of Covariance - Age and Sex with Weight as cofactor

Source of Variation	Significance
Sex	N.S.
Age	N.S.
Dose	***
Age - Sex	N.S.
Age - Dose	N.S.
Sex - Dose	N.S.
Weight	***

produced an average M.S. 2.5 in unimmunized mice. Control infectivity titrations in unimmunized mice, and in surviving allantois-on-shell, were performed on the challenge virus both before and after the operation of intranasal inoculation of the immunized mice. No loss of infectivity occurred.

Table V - 2 shows the mean M.S. of each age - sex combination at each dose level. Detailed results of the outcome of challenge and the allocations of mice to weight classes are given in Table M, Appendix 2. Analysis of covariance was again performed, and is shown in Table N. Pre-immunization weight was again a highly significant factor affecting the outcome of challenge, smaller mice showing a more severe response than larger. Elimination of the effect of weight reduced the apparent influence of age and sex of mice to insignificant levels.

Planned challenge tests for immunogenicity

The information which had been obtained concerning the various factors influencing the outcome of challenge tests in normal and immunized mice, was then used in the planning of experiments to measure the immunogenicity of influenza virus vaccines.

A. A/Swine Vaccines, first experiment

Male W.E.H. mice were segregated after weaning at 4 weeks and were transferred to a thermostatically controlled 15°C

room when 6 weeks old. A constant record of the ambient temperature in this room showed that it ranged between 14.5 and 15.5°C. The relative humidity ranged between 50 and 70% corresponding with the temperature fluctuations, which were due to the regular but intermittent operation of a cooling plant. Fans were strategically placed to ensure a constant air circulation around and into the mouse containers. When the mice reached the age of 9 weeks they were weighed as quickly as possible, and the mean and standard deviation of the weight distribution calculated. The animals with weights less than one standard deviation from the mean were allocated six to a cage, so that each cage had not only approximately the same mean weight but also a full range of the weights. In this way it was hoped to disclose any subtle effects which might be attributable to minor weight variations.

Two inactivated vaccines were prepared; using formaldehyde (Vaccine B), and ultraviolet irradiation (Vaccine C), and following the methods given in Appendix 1. Two-fold dilutions of each vaccine in calcium magnesium saline were prepared, containing a range of 320 to 1280 haemagglutinin units per ml. The vaccines had been titrated at the time of inactivation, three months earlier, and were subsequently found to have suffered no loss during storage, when re-titrated immediately before the preparation of the dilutions. Each vaccine was inoculated using the intraperitoneal route, in 0.25 ml. doses so that challenge could be performed according to the following plan : -

TABLE V - 3

Challenge tests in normal mice to establish the M.S. 2.5 of stock A/Swine virus.

Average M.S. of 10 mice challenged with each dilution

	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Experiment 1 (Preliminary Titration)	-	4.55	3.95	2.63	0.97	0.45
Experiment 2 ø	5.00	4.80	3.89	2.45	0.68	-

ø Control titration carried out in conjunction with challenge of immunized mice.

Dose of vaccine for each vaccine. Haemagglutinin units in 0.25 ml.	Number of mice to be challenged with stock virus diluted to contain		
	1000 M.S.2.5	316 M.S.2.5	100 M.S.2.5
80	6	6	6
160	6		6
320	6	6	6

There were insufficient animals within the specified weight range to allow a complete range of vaccine doses and of challenges and the two groups which might have received 160 haemagglutinin units of the vaccines and undergone challenge with 316 M.S. 2.5 of virus were missing. After completion of the injection schedule, the vaccine dilutions were re-titrated to ensure that no loss of haemagglutinin titre had occurred during the mouse inoculations.

All mice were bled by the retro-orbital technique on the 19th day after immunization, and were challenged 2 days later using stock A/Swine virus diluted in standard medium. To establish the M.S. 2.5 of the stock A/Swine challenge virus a preliminary titration had been performed using the mice which were either too heavy or too light to be used in the test proper, since it had already been established that weight of mice was not a factor influencing the outcome of A/Swine infection in unimmunized mice. The results of the preliminary titration and the control titration for the experiment can be seen in Table V - 3. On the basis that a dilution of 10^{-5} contained the average amount of

TABLE V - 4

Immunogenicity of A/Swine Vaccines

Average M.S. of six mice immunized with formaldehyde and ultra-violet irradiation inactivated vaccines at three dose levels. Challenge with graded doses of stock A/Swine virus.

Dose of Vaccine Haemagglutinin Units	Vaccine B			Vaccine C		
	Challenge Dilution			Challenge Dilution		
	$10^{-2.0}$	$10^{-2.5}$	$10^{-3.0}$	$10^{-2.0}$	$10^{-2.5}$	$10^{-3.0}$
80	2.77	2.35	1.80	2.77	2.12	1.62
160	2.93	-	1.40	2.42	-	1.95
320	2.48	1.00	0.45	2.07	1.17	1.03

Results of Analysis of Covariance omitting all mice immunized with 160 haemagglutinin units of vaccine. Challenge level, Vaccine type, and Dose of vaccine with Weight as cofactor

Source of Variation	Significance
Challenge	*
Vaccine	N.S.
Dose	*
Challenge - Dose	N.S.
Challenge - Vaccine	N.S.
Dose - Vaccine	N.S.
Weight	N.S.

virus which would produce an M.S. 2.5 in mice, the three dilutions used to challenge the immunized mice were 10^{-3}, $10^{-2.5}$ and 10^{-2}. Control infectivity titrations of the virus content of the 10^{-3} challenge dilution were carried out in surviving allantois-on-shell both prior to and at the conclusion of the mouse inoculations, and there was no loss of infectivity. As indicated above a control titration in unimmunized mice was performed after all the immunized mice had been challenged.

Detailed results of the outcome of the challenge are given in Table O of Appendix 2, and the arithmetic means for each dose level are shown in Table V - 4. It is obvious that, with only six mice in each group, the variation in individual response was too great to allow meaningful differentiation of the two-fold increments of vaccine dose. The results of challenge in the mice inoculated with 80 and 320 haemagglutinin units were submitted to analysis of covariance with pre-immunization body-weight (to the nearest gram) as cofactor, and the details of this operation are presented in Table P of Appendix 2.

The significant difference which was found between the results obtained at the three challenge levels was to be expected, since these ranged from 100 to 1000 M.S. 2.5. However the absence of a significant interaction between challenge level and vaccine dose indicated that, within the range of 100 to 1000 M.S. 2.5, variations in the actual level of challenge virus would not invalidate the

results of immunogenicity assays.

Although small variations in body-weight did not affect the outcome of challenge significantly, the average M.S. for animals heavier than the mean weight was 1.65 as compared with 1.90 for mice which were lighter than the mean. It was therefore decided that in future experiments mice whose weights were WITHIN only two-thirds of one standard deviation of the mean would be used.

A significant difference in the outcome of challenge was demonstrated as a result of the response of the mice to vaccine doses which contained four-fold differences in concentration. It is clear from Table V - 4 that challenge of the mice had not differentiated between two-fold increments in vaccine dose.

The problem posed by the large individual mouse variation, clearly indicated by the residual mean square of 2.84, showed that a larger number of mice would be required at each dose level to increase the precision of the challenge tests, and a further experiment with the same vaccines was carried out using ten mice at each dose level.

B. A/Swine Vaccines,
second experiment

In this experiment, 24 weeks old male W.E.H. mice were weighed and all animals more than two-thirds of one standard deviation from the mean were separated and reserved for the preliminary and control titrations in unimmunized mice. The remaining animals, whose overall weight range was only seven grams,

TABLE V - 5

Immunogenicity of A/Swine Vaccines

Individual M.S. of mice immunized with formaldehyde and ultra-violet irradiation inactivated vaccines at three dose levels. Challenge with 100 M.S. 2.5 of stock A/Swine virus.

	Dose of Vaccine B Haemagglutinin Units			Vaccine C Haemagglutinin Units		
	10	20	40	10	20	40
Individual M.S.	4.5	3.0	0.1	4.5	0.3	0.3
	0.8	1.5	0.3	2.8	4.0	4.5
	4.5	2.0	0.1	2.8	4.0	1.5
	0.5	1.5	0.1	2.0	4.5	3.0
	0.5	1.5	0.5	4.5	0.3	1.0
	4.0	2.0	0.5	4.5	0.1	1.5
	1.0	1.8	0.5	4.0	4.5	4.0
	4.5	1.5	0.3	4.5	0.3	0.5
	4.5	1.8	2.5	1.0	0.3	0.5
	2.8	0.8	0.5	0.0	2.0	0.0
Means	2.76	1.74	0.4	3.06	2.03	1.68

Analysis of Variance

Source	d.f.	Sums of Squares	Mean Square	Variance Ratio	Significance
Dose	2	32.6083	16.3041	7.2754	**
Vaccine	1	4.9881	4.9881	2.2258	N.S.
Dose-Vaccine	2	2.3804	1.1902	0.5311	N.S.
Residual	54	121.0130	2.2410		
	59	160.9898			

were distributed in groups of five to cages so that each cage contained approximately the same mean weight and also the same range of weights. Numbering and identification of mice were again carried out, and all cages were transferred to the controlled temperature ($15^{\circ}C$) room as in the previous experiment. When the mice had been acclimatized for one week, two-fold dilutions of the inactivated A/Swine vaccines in calcium magnesium saline were made so as to contain 10, 20 and 40 haemagglutinin units in 0.25 ml. Two cages (10 mice) were injected intraperitoneally with 0.25 ml. of each dilution of the vaccines, and the haemagglutinin levels of the dilutions were then re-checked to ensure that no loss of titre had occurred during the vaccination operation. All animals were bled at 19 days post-inoculation, and two days later were all challenged with 100 M.S. 2.5 of stock A/Swine virus (a 10^{-3} dilution in calcium magnesium saline). Results in individual mice and the means for each dose level are presented in Table V - 5. Analysis of variance of these results is also given and the effect of two-fold increments in dose of vaccines was shown to be significant at the 1% level when ten mice were immunized with each dose. The average mouse score for animals above the mean weight was slightly greater than for lighter animals, indicating that if only those animals weighing within two-thirds of one standard deviation of the mean were used, body-weight could be ignored as a factor influencing the outcome of challenge of immunized W.E.H. mice.

C. A/2/Singapore/1/57M vaccines

Another challenge test was performed using mice of one sex which were transferred to a 15°C environment after numbering and identification. One week was allowed for acclimatization, and then groups of twelve female W.E.H. mice were inoculated with each of three doses of two vaccines prepared from a suspension of A2/Singapore/1/57M virus. For the preparation of the vaccines the same suspension of virus was inactivated by means of the careful addition of sulphur mustard, as described in Appendix 1, and there was no loss of haemagglutinin titre with either preparation. They were designated F1 and F2.

The animals used were twelve weeks old and were distributed to dose levels on a basis of equal body-weight. The two vaccines diluted with calcium magnesium saline to contain 100 haemagglutinin units were injected into mice weighing 30 to 33 g. Two other dilutions containing 200 and 400 haemagglutinin units were injected into mice weighing 33 or 34 g. and 34 or 35 g. respectively. The haemagglutinin contents of the dilutions of the vaccines were checked both prior to and at the conclusion of the inoculations to ensure that no loss of titre had occurfed. A small sample of blood was removed from each mouse on the 19th day after immunization.

A preliminary titration in mice which were heavier than 35 g. established that a dilution of $10^{-3.5}$ of the stock A2/Singapore/1/57M virus produced one M.S. 2.5 in normal W.E.H. mice,

TABLE V - 6

Immunogenicity of A2/Singapore/1/57M Vaccines

Individual M.S. of mice immunized with two sulphur mustard inactivated vaccines at three dose levels. Challenge with 100 M.S. 2.5 of stock A2/Singapore/1/57M virus.

	Dose of Vaccine F-1 Haemagglutinin units			Dose of Vaccine F-2 Haemagglutinin units		
	100	200	400	100	200	400
Individual M.S.	0.8	0.3	0.0	1.0	0.8	0.8
	0.1	0.3	2.0	1.3	0.8	0.5
	4.0	2.5	0.8	0.8	1.3	0.1
	1.5	2.5	0.1	0.5	0.1	0.0
	4.5	0.0	0.5	2.0	1.0	0.1
	0.1	0.8	0.8	1.3	0.3	0.8
	0.5	1.5	0.8	2.5	0.1	0.1
	4.0	1.8	0.8	2.8	4.0	0.5
	0.3	0.1	0.5	0.3	0.3	0.3
	2.5	0.8	1.5	4.5	1.5	1.0
	2.5	1.0	0.0	0.8	0.5	1.0
	1.5	0.1	2.0	2.3	1.5	1.3
Means	1.86	0.98	0.82	1.68	1.02	0.54

Analysis of Variance

Source	d.f.	Sums of Squares	Mean Square	Variance Ratio	Significance
Dose	2	15.0169	7.5084	6.7207	**
Vaccine	1	0.3472	0.3472	0.3108	N.S.
Dose-Vaccine	2	0.3186	0.1593	0.1426	N.S.
Residual	66	73.7367	1.1172		
Totals	71	89.4194			

and challenge of the immunized mice was carried out with a $10^{-1.5}$ dilution on the 21st day after vaccination. A control titration of the virus in unimmunized mice (weighing less than 30 g.) was carried out at the same time and stability of the infectivity of the challenge virus was demonstrated by titration in surviving allantois on shell, both before and after the intranasal inoculation in mice.

The results of this experiment are presented in Table V-6 and show that the demonstration of significant differences between two-fold dose increments of A2/Singapore/1/57M vaccine is reproducible.

Discussion

The finding that pre-immunization body-weight had a highly significant influence on the outcome of challenge in mice immunized with influenza virus vaccines allows the removal of a large source of variation from assays of these vaccines in random bred mice. With A/Swine vaccine, significant effects attributable to age and sex of the animals were reduced to not significant levels when the effect due to weight was eliminated by means of analysis of covariance. However, minor differences in pre-immunization body-weight were not found to influence the results of planned immunogenicity assays, which were performed under carefully controlled environmental conditions at an ambient temperature of 15°C. The effect of body-weight on the outcome of challenge was only observed with immunized mice and, as was

demonstrated in Chapter III, did not occur with challenge of normal mice. Therefore, since the weights of random bred mice are normally distributed, it is possible to use the larger number of animals whose weights are close to the mean for challenge of vaccinated mice whilst heavier and lighter animals may be employed in the necessary control virus titrations.

Further analyses of covariance, which are not detailed here, were performed on the results of both A/Bel and A/Swine vaccine experiments reported in this chapter, this time using pre-challenge body-weight as covariate. Although the effect of pre-challenge weight of mice was still highly significant, the variance ratio in both cases was somewhat smaller than for pre-immunization body-weight. This would indicate that the weight of the mice at the time of inoculation has a fundamental bearing on the type or the extent of the immune response of mice to influenza virus vaccines.

Despite careful control of both the environment and the weights of the animals, there was still a large variation in the response of the immunized W.E.H. mice to challenge. To overcome the effect of this variation it is necessary to use at least ten mice for each vaccine dilution if two-fold differences in immunogenicity are to be measured to a reasonable level of accuracy. This difficulty may subsequently be overcome if it is found that inbred lines of mice or F1 hybrids can be used for challenge with

influenza viruses. Attempts to use C57Bl mice in two immunogenicity assays with A2/Singapore/1/57M vaccines which are not detailed here, were complete failures. However, an assay with B/Lee vaccines in these mice which will be reported in the next chapter, was quite satisfactory. These three experiments were carried out in a 15°C environment, and the possibility of cross-infection with influenza viruses in C57Bl mice at that temperature has already been indicated.

Summary

1) Factorial experiments showed that the response to challenge in influenza virus vaccinated mice of different ages and both sexes was influenced by the pre-immunization body-weight of random bred mice to a highly significant degree.

2) With A/Swine vaccine, the influence of age and sex on the outcome of challenge was found to be due to differences in body weight which accompanied the differences in age and sex.

3) The influence of body-weight was not apparent when animals within two-thirds of one standard deviation of the mean weight were used in immunogenicity assays of A/Swine vaccines.

4) The precision of immunogenicity assays of A/Swine influenza virus vaccines in W.E.H. mice was adequate to demonstrate two-fold differences in potency, when ten or more mice were used for each dose of vaccine.

5) Immunogenicity assays of inactivated A2/Singapore/1/57M vaccines were shown to be reproducible.

CHAPTER VI

CORRELATION OF IMMUNOGENICITY WITH OTHER VACCINE CHARACTERISTICS

Apart from their ability to act as immunogens, influenza virus vaccines possess other properties which may be titrated by means of *in vitro* or *in vivo* tests. Demonstration of the existence of a close correlation between immunogenic potency, proved by challenge experiments, and titre obtained in some simpler test, would permit the use of the latter as a method of assay of vaccine immunogenicity. Moreover, since challenge tests of mice immunized with influenza virus vaccines may only validly demonstrate large differences in immunogenicity levels, proof of a correlation between immunogenicity and some other measureable property of the vaccines might permit more accurate assessments of their potency.

The *in vitro* tests which have been developed to assay various characteristics of influenza virus vaccines include haemagglutinin pattern and photometric titrations, enzyme activity and virus-specific complement-fixing antigenicity tests. A series of experiments were carried out to examine the possible correlation between the results of these tests and immunogenicity levels assessed by challenge of immunized mice. In addition, the antibody absorption test of Fazekas de St. Groth and Webster (1961), and a test based on the micro gel precipitin technique of El-Marsafy and Abdel-Gawad (1961) were also included in these comparative studies.

In another series of experiments, the levels of specific complement fixing, anti-haemagglutinin, and infectivity neutralizing antibodies produced in individual mice as a result of vaccination,

were compared with the outcome of subsequent challenge in these animals. In this way in vivo assessment of antigenicity might be shown to possess a correlation with the level of resistance induced by immunization.

A. In vitro assay of influenza virus vaccines

For a study of the relationship between characteristics of influenza virus vaccines which may be measured by in vitro assay, and immunogenic potency demonstrated in mice, it was necessary to prepare a number of vaccines of different virus strains in which it might be possible to demonstrate different levels of immunogenicity. Since the vaccine preparations which were to be compared had to possess the same virus content, and also the same levels of any other materials which might influence the immune response, it was necessary to use the same virus suspensions of each strain, and to use minimal amounts of treatment with chemical and physical agents to ensure complete inactivation.

Materials and Methods

Preparation of vaccines :- Details of the methods of preparation of the vaccines are given in Appendix 1, and only a brief summary and discussion of the operations is included in this chapter. Purified A/Swine, A/Bel, and B/Lee virus suspensions were prepared from the allantoic fluids of eggs which had been inoculated with 10^3 ID_{50} of these viruses and incubated for two to three days at 35°C.

Purification and concentration were achieved by one cycle of absorption to and elution from human erythrocytes, followed by two

cycles of differential centrifugation. Final re-suspension was in calcium saline to which 0.08% sodium azide had been added. The virus suspensions, and the prepared vaccines, were always stored at 4°C.

The vaccines and their methods of preparation were as follows : -

A) Untreated virus suspensions.

B) Formaldehyde. Formalin (36% w/v Formaldehyde) was added to 0.025% concentration and the suspensions were incubated at 37°C for eight hours.

C) Ultra-violet. Ultra-violet irradiation of the suspensions was carried out for three minutes in a shallow tray at a distance of 20 cm. from a 30 watt germicidal lamp. This provided an intensity of irradiation approximately equal to 270 μ watt/cm.^{2}. Mixing without frothing was achieved by gentle rocking at a rate of ten movements per minute.

D) Heat. Inactivation was achieved by immersion of the suspensions in a water bath, but the time and temperature levels varied with different strains of virus. A/Swine was heated for 50 minutes at 50°C, and B/Lee and A/Bel suspensions for 60 minutes at 55 - 56°C. As a result of heating, the A/Bel suspension suffered a four-fold drop in haemagglutinin titre, which was restored to the original level by exposure of the vaccine to sonic vibrations for two minutes at 5°C., using a Mullard Ultrasonic Drill Generator (50 watt, peak output at 16 kilocycles/second).

E) Mercurial. Metephen (4-nitro-anhydro-hydroxy-mercury-

orthcresol) was added to 1/5000 concentration, and the vaccines were allowed to stand at room temperature for 16 hours. (Ogasawara et al. 1961).

F) Mustard. Sulphur mustard (bis(2 chloroethyl) sulphide) was added with stirring to a concentration of 0.01M (Fong and Bernal, 1953). Stirring was continued for one hour at room temperature, and 0.02M imidazole was then added and allowed to react with any free sulphur mustard which remained in the vaccine (Davis and Ross, 1947).

Each A/Bel and A/Swine vaccine, except the untreated suspensions, was tested for loss of infectivity using inoculation of a 10^{-2} dilution into the allantoic cavity of four chicken embryos (eleven days old) which were subsequently incubated at 35°C for 48 hours. The harvested allantoic fluids, which were all negative for haemagglutinin, were inoculated into further eggs which were also incubated at 35°C. After 48 hours the allantoic fluids from these second passages were all negative. The untreated B/Lee suspension was similarly shown to have lost all infectivity as a result of storage at 4°C for three months before preparation of the vaccines. No infectivity tests were therefore necessary for the "inactivated" preparations of this virus.

Hyperimmune sera: Hyperimmune rabbit sera were prepared by the intramuscular injection of 10000 haemagglutinin units of each strain of virus which had been emulsified with Freund's incomplete adjuvant (Difco) in a VirTis homogenizer at 25000 r.p.m. for five minutes at 0°C. After a lapse of three months, each rabbit was given a single

intravenous injection of 5000 haemagglutinin units of the same virus strain which it had previously received, and was bled two weeks later.

Hyperimmune mouse antibodies were prepared using the method of Anacker and Munoz (1961), and employing three overweight male mice (50 or more g.) for each strain of virus. Freund's complete adjuvant (Difco) was emulsified with 10,000 haemagglutinin units by rapid mixing in a syringe, and the mixture was inoculated intraperitoneally into mice on three occasions at four day intervals. A further dose of mixed virus and adjuvant was given by the same route five weeks later, and considerable distention of the mice occurred during the following five days. Ascites fluid was drained from the peritoneal cavities on the 7th and 14th days after the last injection of vaccine, allowed to clot, and was spun clear and stored at -20°C.

Methods of assay: - Details of the methods used for assay of certain properties of influenza virus vaccines have already been given in Chapter II or will be described in Appendix 1. The following is a brief summary and discussion of these methods :

A) Haemagglutinin Pattern Tests : - These were performed according to the technique of Fazekas de St. Groth and Graham (1955) which has been described in Chapter II.

B) Haemagglutinin Photometric Assay : - The modification of the CCA test which was described by Miller and Stanley (1944) was used with a standard A/PR8 vaccine supplied by the Division of Biologics Standards, Maryland, U.S.A. This method was also described in Chapter II.

C) Enzyme Activity Test : - This was based on the rate of sialidase activity on sialyl-lactose, and the technique was given in Chapter II. The units were again the optical density readings obtained with an Aminoff (1959) test, read in a Beckmann Spectrophotometer, and multiplied by 100. They were distinctive for each strain of virus used in the vaccines.

D) Complement Fixation : - The dilution of each vaccine which combined with 4 units of the strain specific hyperimmune rabbit serum to fix $3HD_{50}$ of complement was estimated using the overnight fixation procedure of Fazekas de St. Groth et al. (1958) which is described in Appendix 1. For each strain, vaccines were diluted to ensure that equal levels of haemagglutinin were present in the primary dilutions.

E) Gel Precipitin : - These assays followed the quantitative micro tube gel precipitin technique of El-Marsafy and Abdel-Gawad (1961), except that the appropriate rabbit hyperimmune antibody was incorporated in the melted agar before preparation of a 2 cm. agar column in the capillary tubes. Vaccine preparations, containing 5000 units of haemagglutinin, were tested by injecting into one end of the capillary, which was then sealed at both ends, and the advance of the leading edge of the main precipitin band was measured to the nearest 0.1 mm. (Augustin et al. 1958) by means of a microscope fitted with a graduated stage.

F) Antibody absorption : - Two methods, equilibrium filtration and equilibrium centrifugation, were used for the assay of the antibody absorbing power of vaccines. Hyperimmune rabbit or

mouse ascites were treated with Receptor Destroying Enzyme as described in Appendix 1, and diluted to 1/40 with calcium saline. Each vaccine was diluted in calcium saline to contain 2000 units of haemagglutinin per ml. and 1.2 ml. was mixed with 1.2 ml. of antiserum, and allowed to attain equilibrium at room temperature for 90 minutes. Ultrafiltration, with 50 mμ Millipore membranes, was carried out using the method and apparatus described by Fazekas de St. Groth and Webster (1961). For equilibrium centrifugation the mixtures of virus and antiserum were spun at 25,000 r.p.m. in micro tubes, using the 30 rotor of a Spinco preparative ultracentrifuge. The micro tubes, of 5 mm. diameter, were fitted into a polystyrene holder, and the average acceleration in individual tubes ranged from 37,000 to 48,000 g.

The filtrates and supernatant fluids were then titrated for anti-haemagglutinin and for neutralizing antibody in the surviving allantois-on-shell system, using the methods described in Appendix 1.

G) Immunogenicity: - The immunogenicities of vaccines were compared using three doses (two-fold increments) in mice of a single age and sex. For a particular dose of each vaccine the weight variation of mice was limited to three grams. All animals were held in an insulated room at 15°C, with constant air circulation around and into the cages. As mentioned in Chapter IV, the relative humidity varied with the regular but intermittent operation of a cooling plant, and ranged from 50% to 70% .

TABLE VI - 1

Comparison of immunogenicity assay of A/Swine vaccines with other methods of titration.

Immunogenicity Assay Average M.S. for ten mice immunized with A/Swine vaccines and challenged with 100 M.S. 2.5 of stock A/Swine virus				Other Methods of Assay				
Vaccines Methods of Preparation	Dose of vaccine in haemagglutinin units			Hyperimmune Rabbit Antibody Absorption Test. Anti-haemagglutinin after Filtration Reciprocal titre ∅ $Log._{10}$	Gel Precipitin Test, Distance in mm. Read at 7 Days	Complement Fixation Test. Reciprocal Antigen Titres $Log._{10}$	CCA units	Enzyme units
	10	20	40					
Untreated	2.76	1.87	1.07	2.77	11.1	2.95	1444	137
Formaldehyde	2.76	1.73	0.84	2.83	11.1	2.80	--	106
Ultraviolet Irradiation	3.06	2.03	1.28	2.83	11.0	2.71	1568	122
Heat	2.62	1.64	0.98	2.80	11.0	2.61	994	40
Mercurial	2.81	1.72	1.30	2.83	11.2	2.64	705	85
Sulphur Mustard	2.70	1.76	1.08	2.77	11.0	2.65	1482	118

∅ Unabsorbed serum 3.21. Essentially the same results with antibody absorption by centrifugation.

The vaccine dilutions were prepared in calcium magnesium saline, and were each inoculated intraperitoneally into ten mice after these had been acclimatized at 15°C for one week. After all the animals had been inoculated, each dilution of virus vaccine was re-titrated to ensure that no loss of haemagglutinin had occurred during the process. Challenge with 100 M.S. 2.5 of stock virus was carried out on the 21st day after immunization, and times of death and extent of pulmonary lesions at autopsy seven days after challenge were used to assess mouse scores as before.

The infectivities of all challenge suspensions were checked before and after the mouse inoculations, using the surviving allantois-on-shell system of Fazekas de St. Groth and White (1958). No loss of infectivity occurred during the performance of any test. The infectivities of the stock challenge viruses for mouse lungs were demonstrated by titrations in unimmunized mice at the conclusion of the challenge operations.

Experimental Results

A/Swine vaccines : -

A/Swine vaccines were prepared as described under Materials and Methods, and their immunogenicities were compared using 24 weeks old male W.E.H. mice. Treatment with Metephen (Vaccine E) caused a 30% drop in pattern haemagglutinin test titre, and all the A/Swine vaccines were diluted so that the same three dose levels of haemagglutinin of each vaccine (10, 20 and 40 units) were used for immunization of the mice. The average M.S. at each dose level which resulted from challenge are given in Table VI.- 1, and it is evident

TABLE VI - 2

Comparison of immunogenicity assay of A/Bel vaccines with other methods of titration.

Immunogenicity Assay				Other Methods of Assay				
Average M.S. for ten mice immunized with A/Bel vaccines and challenged with 100 M.S. 2.5 of stock A/Bel virus				Hyperimmune Rabbit Antibody Absorption Test. Anti-haemagglutinin after Filtration Reciprocal titre ∅ $Log._{10}$	Gel Precipitin Test. Distance in mm. Read at 5 days	Complement Fixation Test. Reciprocal Antigen titres. $Log._{10}$	CCA units	Enzyme units
Vaccines Methods of Preparation	Dose of vaccine in haemagglutinin units							
	125	250	500					
Untreated	2.78	1.34	0.78	2.65	9.7	2.84	2250	255
Formaldehyde	3.25	1.21	0.71	2.74	9.7	2.80	2250	255
Ultraviolet irradiation	2.98	1.59	0.68	2.70	9.7	2.74	2250	222
Heat	2.60	1.49	0.76	2.74	9.6	2.85	-	42
Mercurial	2.65	1.22	0.93	2.65	9.7	2.71	-	163
Sulphur Mustard	3.14	1.71	0.78	2.74	9.6	2.80	-	213

∅ Unabsorbed serum 3.25. Essentially the same results with antibody absorption by centrifugation.

that there were no significant differences in the immunogenicities of the six vaccines.

Each of the vaccines was also titrated by the other methods of assay, and comparative results which corresponded with their immunogenicities, were found for all vaccines except in the CCA and Enzyme tests. Inactivation by heat, or treatment with mercurial compound, resulted in significantly lower titres with these two methods of assay.

A/Bel vaccines : -

The immunogenicities of these vaccines were compared using 12 weeks old female W.E.H. mice. Heat treatment reduced the haemagglutinin titre of the virus suspension by approximately 75%, but, as reported under Materials and Methods, the full titre was restored by means of sonic vibrations. The three levels of haemagglutinin used for inoculation of all vaccines were 125, 250, and 500; challenge with 100 M.S. 2.5 of stock A/Bel virus did not differentiate the immunogenicities of any of the preparations. The mean responses of the mice to challenge, at each level of vaccine, are shown in Table VI - 2.

Titration of the vaccines by the other methods of assay also failed to distinguish between any of the preparations, except that there was very little enzyme activity left in the vaccine which had been subjected to heat treatment to destroy infectivity.

B/Lee vaccines : -

These vaccines were tested for immunogenicity using 24

TABLE VI - 3

Immunogenicities of B/Lee vaccines

Average M.S. for ten mice immunized with B/Lee vaccines and challenged with 100 M.S. 2.5 of stock B/Lee virus.

Vaccines Methods of Preparation	Dose of vaccine Haemagglutinin units 100	200	400
A. Untreated	2.04	1.59	1.34
B. Formaldehyde	2.42	1.62	1.15
C. Ultra-violet Irradiation	3.61	3.48	2.54
D. Heat	3.00	2.56	2.20
E. Mercurial	3.40	2.74	∅
F. Sulphur Mustard	2.35	1.71	1.38

∅ Missing block

Results of Analyses of Variance

Vaccines B. and C

Source of Variation	Significance
Vaccine	***
Dose	*
Vaccine-Dose	N.S.

Vaccines B. and D

Source of Variation	Significance
Vaccine	*
Dose	*
Vaccine-Dose	N.S.

TABLE VI - 4

Immunogenicities of B/Lee vaccines

Average M.S. for ten W.E.H. mice immunized with B/Lee vaccines and challenged with 100 M.S. s.5 of stock B/Lee virus

Vaccines Methods of Preparation	Dose of vaccine Haemagglutinin units 100	200	400
B. Formaldehyde	2.70	1.78	1.19
C. Ultra-violet irradiation	3.58	2.53	2.27
D. Heat	2.86	2.48	2.01
D. Mercurial	3.42	2.50	2.16

weeks old male C57Bl mice, and the dilutions of four of the vaccines had to be adjusted so as to allow for a drop in pattern haemagglutinin titre which occurred with the vaccines which were inactivated by mercurial treatment and by heat. The three levels used were 100, 200 and 400, and the results of the challenge are presented as the means for each dose level in Table VI - 3. The outcome of inoculation with 100 M.S. 2.5 of stock B/Lee virus was found to vary with the type of vaccine preparation used for immunization of the mice. Untreated virus suspension and vaccines inactivated with formaldehyde or sulphur mustard were found to be indistinguishable by immunogenicity assay; but ultra-violet irradiation, and treatment with heat or a mercurial preparation, appeared to have considerably reduced the effectiveness of the virus preparation as an immunogen. Individual data and analyses of variance comparing the formalinized vaccine with both ultra-violet irradiated and heat inactivated preparations are given in Tables Q and R of Appendix 2, and confirm the significance of the different immunogenicities of these vaccines.

The experiment was repeated using immunization of 12 weeks old W.E.H. male mice with four of the vaccines, formalinized, ultra-violet irradiated, heat and mercurial treated preparations. The mean responses to challenge are shown in Table VI - 4, and although there was not a significant difference between the results with heat treated and formalinized vaccines, the ultra-violet irradiated and mercurial treated vaccines were again found to be significantly less immunogenic than the formalin inactivated preparation.

TABLE VI - 5

Titration of B/Lee vaccines by means of gel precipitin, complement fixation, and antibody absorption tests.

Vaccines Methods of Preparation	Gel precipitin test Distance in mm. Read at 5 days	Complement fixation Reciprocal Antigen Titres. Log.10
Untreated	10.5	2.94
Formaldehyde	10.3	2.90
Ultra-violet Irradiated	10.3	2.87
Heat	10.4	2.84
Mercurial	10.3	2.81
Sulphur Mustard	10.3	2.90

Antibody Absorption Tests

Reciprocal titres ($\log_{.10}$) of sera after absorption.

Vaccines Methods of Preparation	Mouse Hyperimmune ascites. Anti-haem-agglutinin after filtration	Rabbit Hyperimmune Serum		
		Anti-haemagglutinin after		Neutraliz-ation of infectivity after Filtration
		Filtration	Centrifug-ation	
Untreated	3.40	3.25	2.92	2.41
Formaldehyde	3.40	3.25	2.92	2.44
Ultra-violet irradiation	3.22	3.25	2.92	2.50
Heat	3.40	3.25	2.83	2.42
Mercurial	3.40	3.42	2.92	2.47
Sulphur Mustard	3.40	3.25	2.80	2.50
Control serum Not absorbed	3.79	3.75	3.28	2.83

Since the vaccines were all inoculated at the same levels of pattern haemagglutinin units, it is obvious that there was no correlation between this property and immunogenicity. In the case of these B/Lee vaccines the relative CCA titres showed close correspondence with the pattern haemagglutinin results, and although the heat and mercurial treated vaccines had lost almost all enzyme activity, the ultra-violet irradiated preparation was still fully active. The results of the gel precipitin, complement fixation, and antibody absorption tests performed on all the vaccines are presented in Table VI - 5, and it is apparent that none of these methods of assay gave results which corresponded with the immunogenicity levels found in the challenge tests in mice.

The results of the investigations of the correlation of *in vitro* methods of titration with immunogenicity differed with the virus strains being tested. Therefore, before equating the immunogenic potency of an influenza virus vaccine with its enzyme activity, gel precipitin or antibody absorbing capacity, complement fixing antigenicity, or its haemagglutinin content, whether this is measured by pattern or photometric test, it will be necessary to separately demonstrate correlation in the case of each particular strain of virus.

B. *In vivo* assay of influenza virus vaccines.

The ability of influenza virus vaccines to act as antigens in mice has been studied using the serum samples which were collected on the 19th day after immunization as referred to in earlier

chapters of this thesis. In that way it has been possible to examine the correlation which exists between serum antibody levels measured in different systems, and the subsequent outcome of challenge in mice.

Correlation between serum antibody level and the outcome of challenge : -

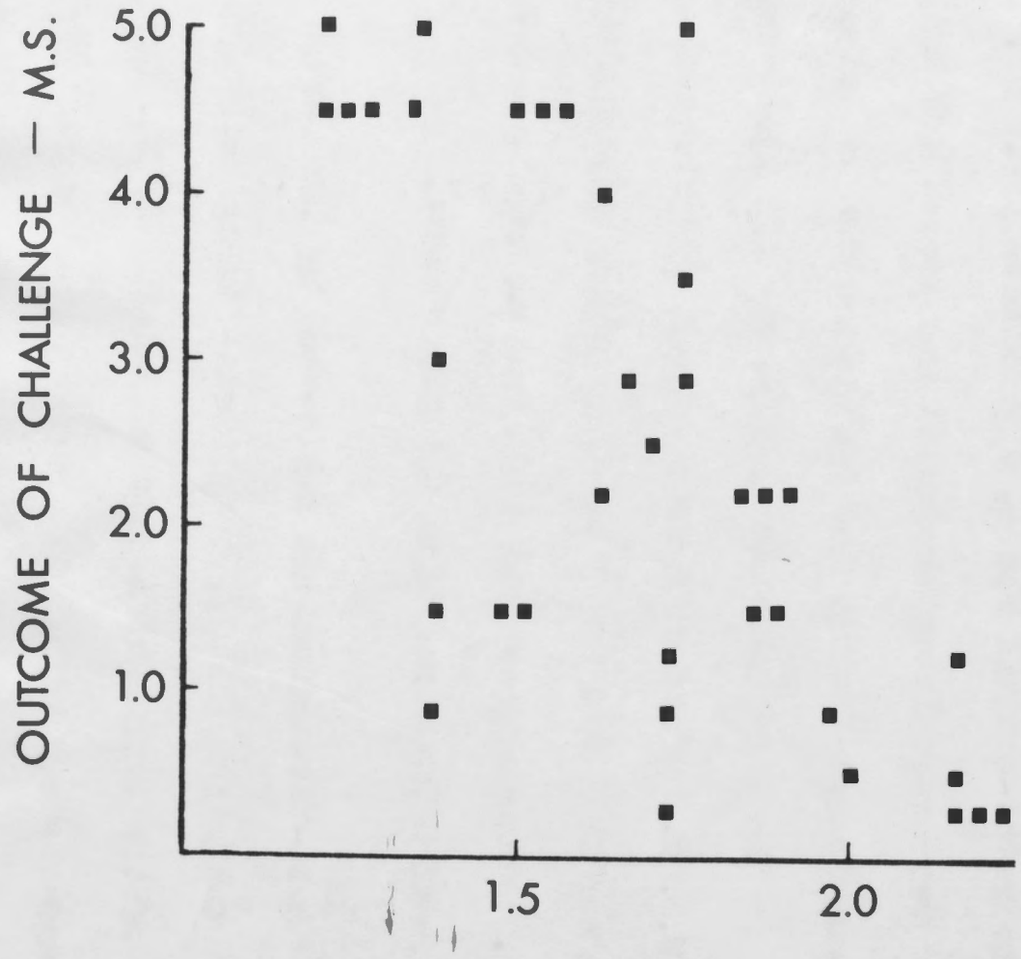
Three two-fold dilutions of A/Swine vaccine, inactivated by 0.025% formaldehyde, were prepared in calcium magnesium saline, and each was inoculated into twelve male W.E.H. mice. The mice were twelve weeks old and weighed between 34 and 36 grams; they had been acclimatized to, and throughout the experiment were held in, a 15°C environment. On the 19th day after vaccination all animals were bled by the retro-orbital route, approximately 0.5 ml. being obtained. Sera were later separated and tested for anti-haemagglutinin, complement-fixing, and neutralization of infectivity content. On the 21st post-inoculation day all mice were challenged with 100 M.S. 2.5 of stock A/Swine virus, and the outcome of infection was followed for 7 days, at which time all survivors were autopsied. Mouse scores were estimated as usual, and were compared with the results obtained with the other tests.

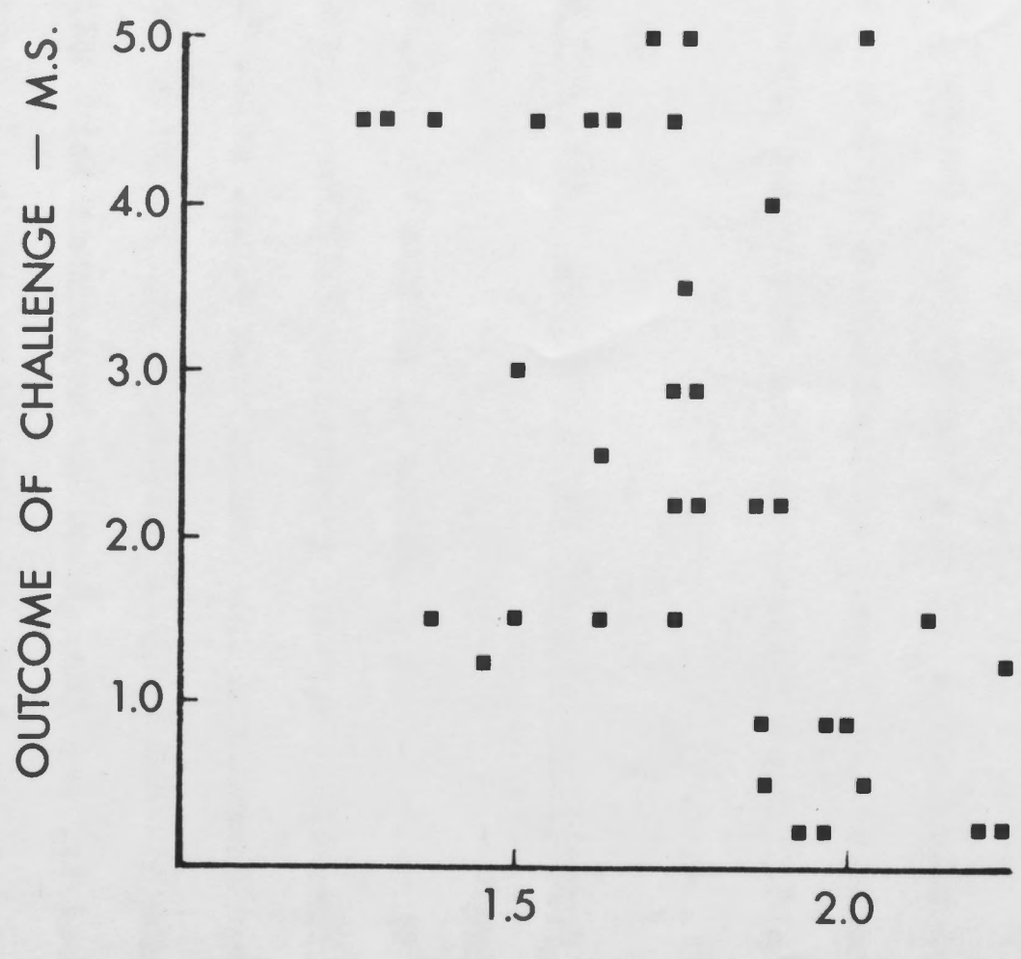
Anti-haemagglutinin was tested by the method of Fazekas de St. Groth and Graham (1955), complement-fixing antibody was assessed by means of the overnight method of Fazekas de St. Groth et al. (1958), and the surviving allantois on shell technique of Fazekas de St. Groth and White (1958) was used to estimate the infectivity

FIGURE VI - 1

Scatter diagrams to show the association between infectivity neutralizing and anti-haemagglutinin antibodies, and the outcome of challenge with A/Swine virus in immunized mice.

(A) Infectivity Neutralizing (B) Anti-haemagglutinin





neutralizing antibody content. All three methods are described in Appendix 1.

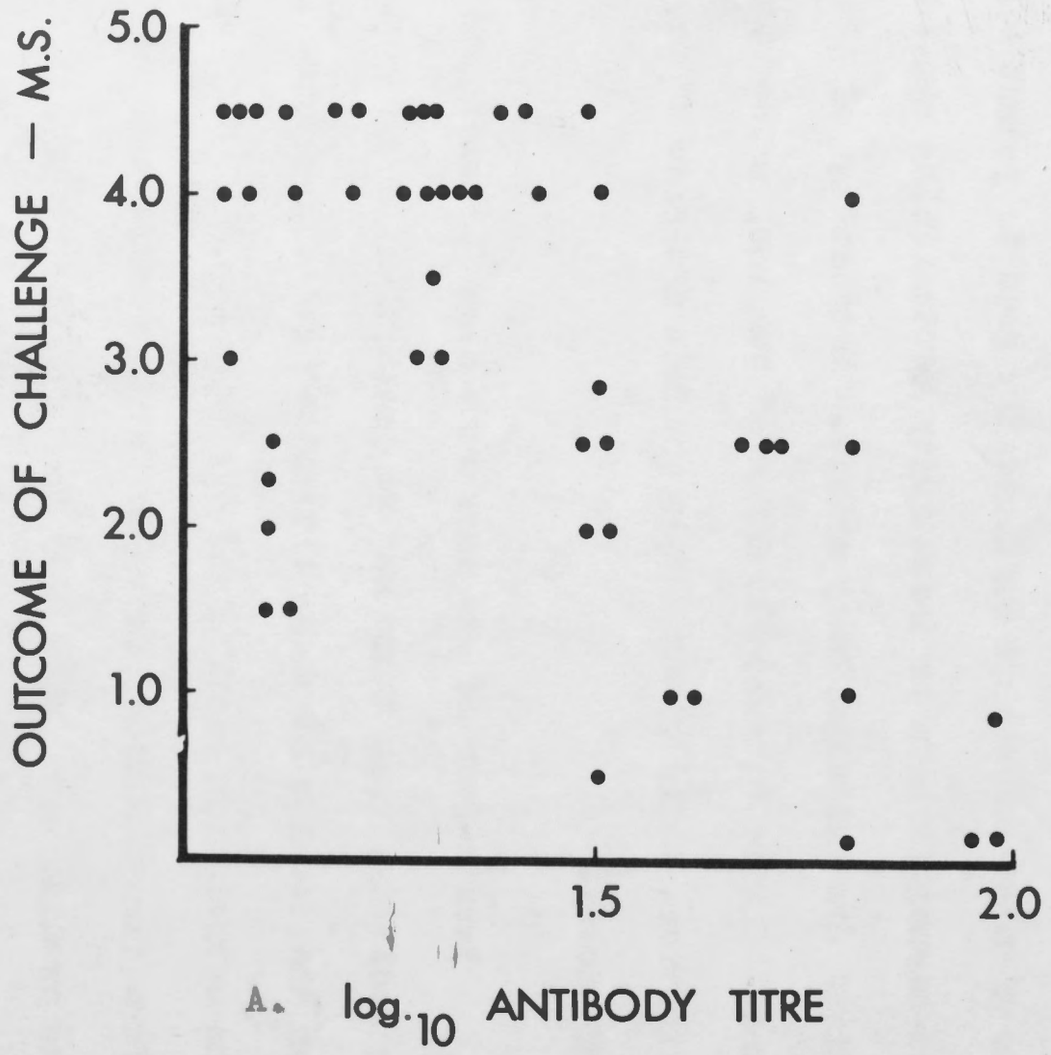
As might be expected, only limited correlation was found for any method of assay and the outcome of infection. Scatter diagrams have been prepared showing the correlations between anti-haemagglutinin and neutralizing antibodies, and are shown in Figure VI - 1. The product-moment correlation coefficient for neutralizing antibody was -0.68, indicating that 46% of the variation in the outcome of challenge could be accounted for by a linear correlation with this measurement of serum antibody. For anti-haemagglutinin the coefficient was -0.52, which indicates a 27% correlation. Whilst they represent moderate degrees of correlation, neither of these levels is sufficiently good to permit substitution of anti-haemagglutinin or infectivity neutralizing antibody titrations for immunogenicity assays. Estimations of the antibody responses of mice to suitable doses of vaccine, using either method of titration, could provide only a crude estimate of vaccine immunogenicity.

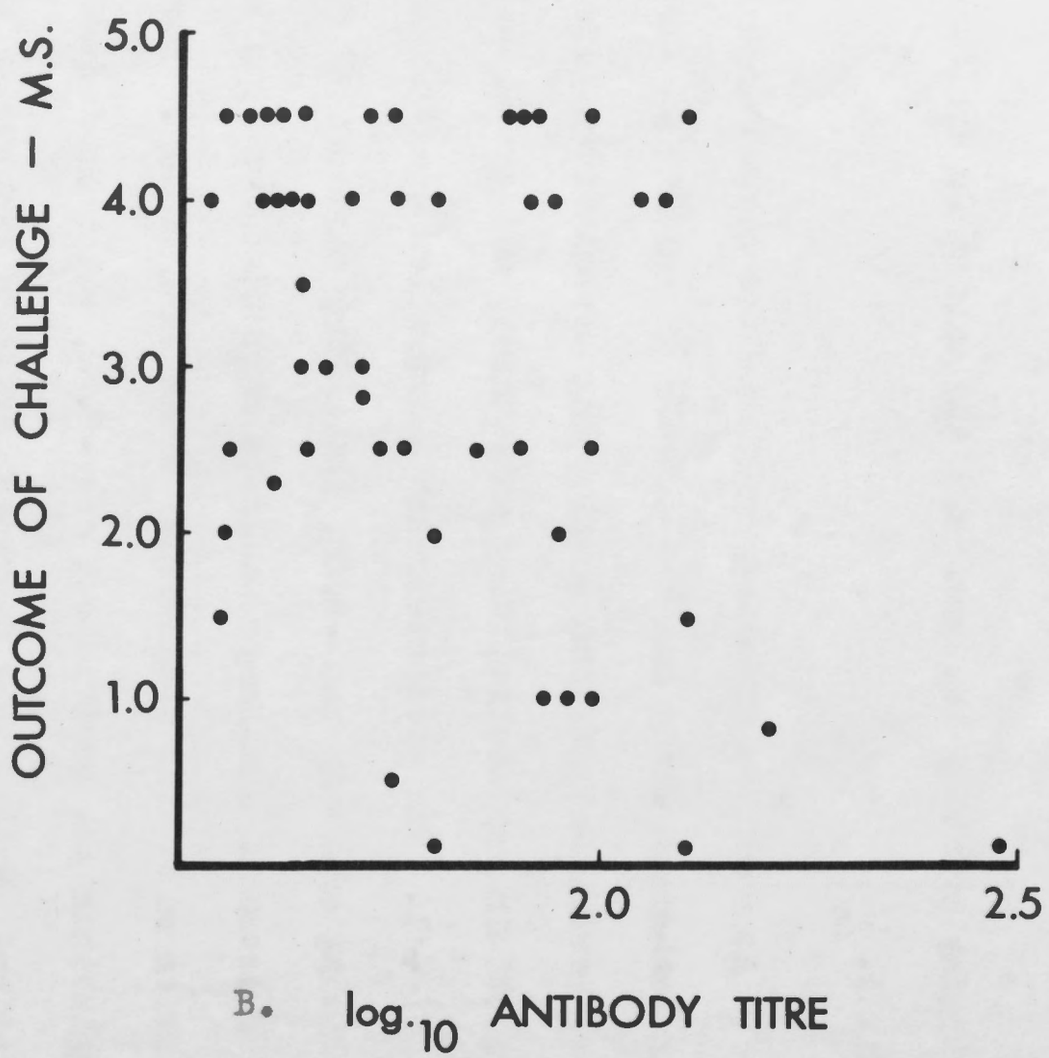
Twenty-four of the sera were also titrated for complement-fixing antibody, but there was no indication of any correlation between the results of these titrations and the outcome of challenge, and the correlation coefficient was only -0.21. This method was therefore regarded as of no value for the estimation of immunogenicity. Similar results were found with the antibody responses of mice to an A2/Singapore/1/57M vaccine, and the lack of good correlation between

FIGURE VI - 2

Scatter diagrams to show the association between the anti-haemagglutinin levels in the sera of mice bled two days prior to and five days after intranasal challenge with B/Lee virus.

(A) Two days pre-challenge (B) Five days post-challenge





antibody titrations and the outcome of challenge limits the value of such assay systems as indicators of immunogenicity.

Effect of the anamnestic response on the outcome of challenge

Fifty male W.E.H. mice, twelve weeks old, were each inoculated with 50 haemagglutinin units of formaldehyde inactivated B/Lee vaccine. The animals weighed between 30 and 36 grams, and were acclimatized at 10 °C for one week before vaccination, and were subsequently held at that temperature during the course of the experiment. On the 19th post-inoculation day, approximately 0.5ml. of blood was taken from each mouse by the retro-orbital route, and two days later all animals were challenged with 100 M.S. 2.5 of stock B/Lee challenge virus. Five days after challenge approximately 0.25 ml. of blood was obtained by the retro-orbital technique, and the experimental influenza in the mice was followed to the end of seven days after challenge, when all survivors were autopsied, and mouse scores estimated.

Sera from the two bleedings were separated and tested for anti-haemagglutinin content by the method of Fazekas de St. Groth and Graham (1955). Scatter diagrams are shown in Figure VI - 2, and the product-moment correlation coefficients for the preliminary and post-challenge sera are -0.59 and -0.37 respectively. Thus, whilst a moderate correlation has been shown once more between the pre-challenge level of anti-haemagglutinin in serum and the outcome of infection, the level of antibody five days after challenge has only a slight relationship with the ultimate fate of the mice.

Discussion

The effect of some inactivating procedures on several properties of influenza virus vaccines varies with different strains of virus. In the work reported here, ultra-violet irradiation or treatment with a mercurial compound of a B/Lee vaccine, which had ceased to be infectious for chicken embryos as a result of standing for three months at 4°C, produced a marked loss of immunogenicity. A lowering of immunogenic potency was also seen with heat treatment of this vaccine, although the reduction was not quite so large. These treatments had no effect on the immunogenicities of A/Swine and A/Bel suspensions, although their infectivities for chicken embryos were completely removed. Haemagglutinin titres, precipitin and complement fixing antigen, as well as antibody absorbing capacity, were not affected by any of the inactivation procedures. Heat treatment, of course, destroyed most of the enzyme activity of the virus suspensions, and addition of the mercurial compound also had that effect on A/Swine and B/Lee vaccines, but not on A/Bel. Henle and Henle (1947) found that ultra-violet irradiation of an A/PR8 suspension led to a rapid loss of immunogenic potency for mice, and that this was not accompanied by a corresponding drop in haemagglutinin titre. In their hands, a B/Lee virus suspension lost immunogenicity at the same rate as it lost haemagglutinin titre. McLean (1961) has reported on the variable reaction of different A1 strains to inactivating agents, a phenomenon which caused considerable difficulty in the preparation of vaccines of those strains.

The experiments reported here indicate that there is no justification for equating immunogenicity with the results obtained by testing for several properties of influenza virus vaccines, unless a good correlation has been shown between that property and immunogenicity of a particular strain of virus. Of the tests for antigenicity, the method of measuring infectivity neutralizing antibody using the surviving allantois on shell technique was found to give the closest correlation with immunogenicity. This is in keeping with the findings of Webster (1962) who studied the different antibody responses of rabbits to influenza virus vaccines, and showed that this neutralizing antibody test measured predominantly the best quality antibody present in any serum. In this respect it was superior to both the anti-haemagglutinin and complement fixation tests, although the level of correlation was not really satisfactory. From the practical aspect of vaccine preparation, where immunogenicities may not need to be known with more than reasonable accuracy, a suitable infectivity neutralizing antibody titration might suffice.

The demonstration that the boosted level of antibody which follows infection of immunized mice did not show as good correlation with the outcome of challenge as the pre-challenge level, indicates that the level of antibody in the respiratory tract at the time of infection is the most important factor in the preventive activity of antibody against influenza viruses. In some cases good secondary responses in the mice did not save them from severe pulmonary

consolidation and death. This is in keeping with the earlier work of Fazekas de St. Groth and Donnelley (1950b) in which they showed a correlation between antibody levels in the bronchi and the outcome of challenge in mice. The antibody levels in the bronchi of the mice were also related, to some extent, with levels of antibody in the corresponding sera.

Summary

1) The immune response of mice to inoculation with influenza virus vaccines which had been subjected to different chemical and physical treatments has been assessed by means of intranasal challenge.

2) The immunogenicity levels produced by the vaccines were compared with the results of titrations of various *in vitro* tests performed on the vaccines.

3) Enzyme and CCA titres showed very little correlation with the immunogenicity of vaccines prepared from all virus strains, but pattern haemagglutinin titre, complement fixing antigenicity, gel precipitin function, and antibody absorption results by several techniques, also failed to correspond with a marked loss of immunogenicity which occurred with some treatments of a B/Lee suspension.

4) The infectivity neutralizing antibody levels of pre-challenge sera showed better correlation with the immune status of vaccinated mice than did the anti-haemagglutin levels. Measurement of the virus complement fixing antibody level was not significantly correlated with the outcome of challenge.

5) The boosted antibody response in immunized animals which

followed infection did not appear to influence the outcome of challenge in vaccinated mice.

CHAPTER V11

GENERAL SUMMARY AND CONCLUSIONS

The immunogenicity of influenza virus vaccines has been investigated, using intranasal challenge of immunized mice to assess the levels of resistance induced by vaccination. The need for adequate preliminary biological studies before attempting to set up assay systems of this type was demonstrated by the discovery of a number of factors which may have a marked influence on either the response to vaccination or the outcome of infection with influenza viruses.

As well as ensuring the basic requirements of adequate food and water, and access to a normal atmosphere, it was found that a stable environment had to be provided for mice which were to be used in intranasal challenge experiments with influenza virus. Reactions to infection were increasingly severe in an inverse relationship to the ambient temperature of the environment. Accordingly, challenge tests were carried out at relatively low environmental temperatures which allowed maximum sensitivity to virus infection, but were still compatible with the health and general well-being of the animals as indicated by continued gain in body-weight. It was essential, however, to acclimatize the mice for some days before challenge, because the severity of the response was increased if the animals were transferred to a cold environment after infection.

The antibody responses to vaccination, and reactions to infection, of both an inbred and a random bred line of mice were studied. Although the antibody responses were essentially similar,

the particular inbred line which was chosen for testing was found to experience cross-infections with influenza virus when kept in small groups under cool environmental conditions.

Only sexually mature mice were used, and the age of the oldest animals used was six months. Within these limits, no significant differences could be found in the responses to infection of mice of different ages. For a wide variety of virus strains the outcome of challenge was essentially similar in both sexes, but male mice were found to be much more susceptible to B/Lee virus infection than females. Differentiation in response according to sex of the mice was not, however, common to type B viruses, and was not confined to one strain of mice.

The body-weight of mice did not affect the results of challenge in normal animals, but the pre-immunization weights influenced the outcome of infection in vaccinated animals to a very high level of significance. The effect of body-weight was demonstrated using the full distribution of weights which co-exist with a range of ages and both sexes, of a random bred strain of mice, and was not apparent when animals of one age and sex were selected to be within two-thirds of one standard deviation from the mean weight.

The antibody responses of mice to influenza virus vaccines were not affected by a short period of partial dehydration, although this treatment led to an increased mortality when the immunized mice

were challenged. The anti-haemagglutinin antibody response was greater in mice held in a warm as compared with a cold environment, but the difference was not so great that any problem would arise if vaccinated mice were exposed to temperatures which varied within the range normally experienced in animal holding rooms.

Despite the recognition and control of many factors other than dose which influence the outcome of challenge in immunized mice, it was necessary to use at least ten animals for each dose to ensure responses which differed significantly with doubling concentrations of vaccine. The slope of the vaccine dose-response curve was not affected by small differences in the levels of virus used for challenge, providing that the dose of challenge virus was at least 100-fold greater than the amount which would produce a median level of lung damage in unimmunized mice.

The immunogenicities of suspensions of two type A virus strains were not affected by several different chemical and physical methods of inactivating infectivity. Two of these treatments did, however, affect the CCA titre and the enzyme activity of the suspensions, indicating that these methods of titration were unsatisfactory for use as direct measures of vaccine immunogenicity. The immunogenicity of a type B/Lee virus suspension was seriously affected by ultra-violet irradiation or addition of a mercurial compound, with some indication that heat treatment had also lowered its potency. The loss of immunogenicity, in each case, was not

paralleled by a corresponding drop in haemagglutinin or gel precipitin titre, and complement fixing antigenicity and the ability to bind antibody were also unaffected. There is therefore no certainty that measurement of any of these properties will provide a valid assessment of the immunogenicities of influenza virus vaccines.

The virus specific complement fixing antibody levels produced in mice in response to vaccination were also found to have little bearing on the outcome of subsequent intranasal challenge. There was a moderate correlation between the anti-haemagglutinin and infectivity neturalizing antibody levels in serum and the results of infection, and the latter method of titration showed the closest correspondence with the state of immunity produced in mice.

The anamnestic response of anti-haemagglutinin antibody following challenge of immunized mice appeared to have little effect on the outcome of infection. This was in keeping with the finding of Baron et al. (1963) that inhibition of the antibody forming mechanism did not increase the mortalities and lung lesions which followed intranasal infection in mice in comparison with untreated controls.

The fallacy of generalization with regard to the biological properties of influenza viruses has been demonstrated throughout this thesis. The profound influence which Kalter (1949) found of age of Swiss mice on the results of infection with PR8 virus was not seen in the case of A/Swine and B/Lee challenge in a random bred line, and differentiation of the severity of the response to challenge with

sex has only been shown with B/Lee virus. Addition of a mercurial compound to a concentration which destroyed most of the enzyme activity of A/Swine and B/Lee viruses had very little effect on this property in an A2/Singapore/1/57M suspension (Table II - 3), and various methods of inactivating infectivity had different effects on the immunogenicities of different virus suspensions (Chapter VI).

An unexpected finding was that the rate of virus multiplication and the final titre of virus in the lungs of mice infected with large doses of A/Swine virus were not significantly different in three environments which had a profound influence on the mortalities and extent of lung consolidation in mice. The attainment of maximal detectable levels of virus in the lungs coincided with a hypothermic reaction in the animals, which varied with the environment, and was prognostic of the ultimate severity of infection in the animals. There was also no detectable difference in the virus growth in male and female mice inoculated intranasally with B/Lee virus although the strain produced widely differing effects in the two sexes. It is obvious that the outcome of influenza virus infection in mice may be influenced as much by the response of the host as by the extent of virus proliferation.

The recent demonstration that haemagglutinin sub-units, split off from the intact virus by treatment with ether (Davenport, 1961), are effective as immunogens and also non-toxic in humans (Webster, 1963) indicates that these may replace intact virus suspensions in future vaccines. There are no reasons why the

immunogenicities of these more refined products cannot be assessed by the method which has been used in the work reported in this thesis. Should the combination of the haemagglutinin units with an inert mineral carrier in an adjuvant vaccine prove to be a more effective and safe immunogen, it will merely be necessary to change the route of injection for vaccination of the test mice. Friedewald (1944) has shown that, following sub-cutaneous inoculation of adjuvant vaccines in mice, the immunity status of the animals could be assessed by intranasal challenge.

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APPENDIX 1

Materials and Methods

This includes materials and methods which have not been described in the text.

Diluents:

Saline

NaCl	8.5 g.
De-ionised and distilled water to make	1000.0 ml.

Sterilized by autoclaving at 10 lb. pressure for 30 minutes.

Calcium Saline

NaCl	8.0 g.
$CaCl_2 \cdot 6H_2O$	5.0 g.
Phenol Red (Clark and Lubs Indicator)	1.0 ml.
Tris (tris-hydroxymethyl aminomethane)	25.0 g.
HCl (conc.)	20.0 ml.
De-ionised and distilled water to make	1000.0 ml.

Adjusted to pH 7.2 with N/1 HCl. Sterilized by autoclaving at 10 lb. pressure for 30 minutes.

Calcium Magnesium Saline

(Fazekas de St. Groth et al. 1958)

NaCl	8.5 g.
$MgCl_2 \cdot 6H_2O$	0.168 g.
$CaCl_2 \cdot 6H_2O$	0.056 g.
H_3BO_3	1.203 g.
$Na_2B_4O_7 \cdot 10H_2O$	0.052 g.
De-ionised and distilled water to make	1000.0 ml.

Sterilized by autoclaving at 10 lb. pressure for 30 minutes.

Standard Medium

(Fazekas de St. Groth and White, 1958)

NaCl	8.0 g.
KCl	0.60 g.
$MgCl_2 \cdot 6H_2O$	0.10 g.
Glucose	0.30 g.
$CaCl_2 \cdot 6H_2O$	1.60 g.
Chloramphenicol	0.10 g.
Gelatin ϕ	2.00 g.
Phenol Red	1.00 mg.
De-ionised and distilled water to make	1000.0 ml.

Adjusted to pH 7.0 with N/1 NaOH, and sterilized by autoclaving at 10 lb. pressure for 30 minutes.

Citrate Saline (for Bleeding)

NaCl	9.0 g.
Na Citrate	50.0 g.
De-ionised and distilled water to make	1000. 0 ml.

Erythrocytes:

Human Erythrocytes

Human red cells were obtained from the N.S.W. Red Cross Blood Transfusion Service. They were washed and centrifuged three times in saline, and after the final centrifugation the packed cells were stored at 4°C before use.

Fowl Erythrocytes

Fowls were bled from the wing vein into citrate saline. The red cell suspension was centrifuged and washed three times in

saline, and after the final centrifugation a 5% suspension in saline was prepared and stored at 4°C. Sufficient cells were removed and used as required, and for haemagglutinin and anti-haemagglutinin titrations fresh suspensions were prepared every 48 hours.

Sheep Erythrocytes

Sheep were bled from the jugular vein into citrate saline. The red cell suspension was centrifuged and washed three times in saline, and after the final centrifugation an 8% suspension in calcium magnesium saline was prepared and stored at 4°C.

Sera:

Mouse Sera

Mouse blood samples were obtained using the retro-orbital technique of Halpern and Pacaud (1951), using a pasteur pipette with a bevelled tip. The small quantities of blood which were taken were allowed to clot on the side of 3x1/2" glass tubes, and these were then stoppered and placed upright in racks at 4°C. After 72 hours in the cold, sera were pipetted off from the small quantities of red cells which settled to the bottom of the tube. In this way it was possible to avoid haemolysis, whilst collecting the maximum possible amount of serum. All mouse sera were stored at -20°C in small tubes closed with strips of Parafilm plastic compound.

Hyperimmune Sera and Ascites Antibody

The description of the preparation of hyperimmune rabbit

antisera, and hyperimmune mouse ascites antibody are given in Chapter VI.

Other Reagents:

Receptor Destroying Enzyme

This reagent was supplied by Dr. G. L. Ada of the Walter and Eliza Hall Institute, Melbourne. When titrated by the method of Burnet and Stone (1947) the Receptor Destroying Enzyme had a titre of 10,000 units per ml. 50% analytical reagent glycerol was added and the Receptor Destroying Enzyme was stored at -20°C. when not in use.

Haemolysin

Rabbit Haemolysin was supplied by the Commonwealth Serum Laboratories, Parkville, Victoria, and contained 50% analytical reagent glycerol. A dilution of 1/800 in calcium magnesium saline was used to sensitize an equal volume of 8% sheep erythrocytes. The mixture was incubated for 15 minutes at room temperature and the 4% suspension of sensitized cells was stored at 4°C when not in use.

Complement

Lyophilised Guinea Pig Complement was supplied by the Commonwealth Serum Laboratories, Parkville, Victoria, and was reconstituted with distilled water. An equal volume of a solution of 12% sodium acetate and 4% boric acid in distilled water was added (Mackie and McCartney, - 1956). The complement was held at 4°C, and was diluted in calcium magnesium saline for the preparation

of 3 HD_{50} units, as required.

Experimental Animals:

Chicken Embryos

Fertile eggs which had been incubated for nine days were obtained from local suppliers, and were incubated at 38°C for a further period of 48 hours before use.

Mice

Mice were bred in the Australian National University animal breeding establishment, and were of two strains. The inbred C57Bl line originated from the Division of Animal Genetics, Commonwealth Scientific and Industrial Research Organization, Sydney, N.S.W. The random bred W.E.H. line originated from the Walter and Eliza Hall Institute, Melbourne.

Weighing of Mice

Mice were weighed on a Mettler K7T balance, and the pan of the balance was covered by a sheet of aluminium foil. Mice transferred from cages to the covered pan remained stationary during the period in which the balance came to equilibrium, allowing observation of the weight, to the nearest 0.1 g. if required.

Viruses ;

Stock Challenge Viruses

The following egg and mouse adapted influenza virus strains were used:

A/Swine Shope, R. D. (1935)

A/PR8 Francis, T. Jr. (1934)

B/Lee Francis, T. Jr. (1940)

A/Bel Burnet, F. M., and Bull, D.R. (1943)

A2/Singapore/1/57M Egg adapted strain supplied by the W.H.O. Influenza Centre, Mill Hill, London; and adapted to growth in the mouse lung at the Commonwealth Serum Laboratories, Parkville, Victoria.

B/Tas./27/53M Strain adapted to eggs and to growth in the mouse lung at the Commonwealth Serum Laboratories, Parkville, Victoria.

Stock challenge virus suspensions of each of these viruses were prepared by inoculating chicken embryos which had been incubated for eleven days by the allantoic route, using 10^3 ID_{50} of virus per egg. After 36 hours incubation at 35°C, the allantoic fluids were harvested rapidly, without damaging blood vessels, and 20% analytical reagent glycerol was added. The viruses were ampouled and then frozen in liquid nitrogen and stored at -60°C in a mechanical refrigerator.

Preparation of Vaccines

A. Untreated virus suspensions : - These were prepared by inoculating chicken embryos, previously incubated for eleven days, by the allantoic route, with 10^3 ID_{50} of each virus strain. In the case of B/Lee the eggs were incubated at 35°C for three days, and in

all other cases for two days, before harvesting the allantoic fluid. One cycle of absorption to human erythrocytes was carried out at 4°C, using a volume of cells equal to 2% of the total volume of the allantoic fluid. After settling overnight, the cells were completely sedimented by centrifugation, and the supernatant fluid was discarded. Elution was carried out in sterile calcium magnesium saline at 37°C for three hours, and the cells were then spun down and discarded and the supernatant retained. Two further purifications were achieved by two cycles of differential centrifugation using the 21 rotor in a Spinco, Model L preparative ultracentrifuge at an average acceleration of 60,000 g. to deposit the virus. After the final ultracentrifugation the virus was resuspended in calcium saline to which 0.08% sodium azide was added, and the vaccine was stored at 4°C.

B. Formaldehyde: - Formalin (36% w./v. Formaldehyde) was added to 0.025% to the virus suspension prepared as in A. After incubation for eight hours at 37°C, the vaccine was tested for absence of infectivity as described in Chapter VI, and was stored at 4°C.

C Ultra-violet irradiation : - Virus suspensions prepared as in A. were irradiated in 20ml. volumes in an open shallow dish, at a distance of 18cm. from a Phillips Germicidal Lamp (30 watts). The intensity of irradiation was approximately 270 μwatt/cm.^{2} at this distance, and more than 95% of the output of the lamp was at 2537 Å. Gentle rocking of the dish, with 10 movements per minute, kept the

suspensions agitated without causing the formation of foam. The vaccine was stored at 4°C, after testing for the absence of infectivity.

D. Heat : - After preparation as in A. A/Swine virus suspension was immersed in a water bath at 50°C for 50 minutes. Similarly prepared B/Lee, A/Bel, and A2/Singapore/1/57M suspensions were held in a water bath at 55 - 56°C for 60 minutes. Absence of infectivity was demonstrated, and the vaccines were stored at 4°C.

E. Mercurial Compound: - Metephen (4 - nitro - anhydro - hydroxy - mercury - orthocresol) was added to a concentration of 1/5000 to suspensions of virus, prepared as in A. and was allowed to react at room temperature for 16 hours before transfer to 4°C. The compound was supplied as a solution in dilute ethanol, and the final concentration of C_2H_5OH was 2%. The suspensions were tested for absence of infectivity.

F. Sulphur Mustard: - Virus suspensions, prepared as in A. were agitated with a magnetic stirrer, in 20ml. volumes. Then 0.025 ml. of sulphur mustard - bis (2 Chloroethyl) Sulphide - was added, giving a final concentration of 0.01M. Stirring was continued at room temperature for one hour, and 0.027 g. of imidazole was added and allowed to react with any free sulphur mustard which remained. After eight hours stirring at room temperature the vaccines were tested for absence of infectivity and stored at 4°C.

Intranasal Challenge of Mice

Intranasal inoculation

A mixture of two parts anaesthetic ether (containing 0.002% w./v. hydroquinone) with one part chloroform was absorbed in a cotton wool pad and placed at the bottom of a clear cylindrical glass jar, 12 cm. in diameter and 20 cm. in height. A 12 cm. diameter sheet of aluminium foil was cut and placed on top of the anaesthetic pad, and a further pad of cotton wool surrounded by cotton-gauze was placed on top of the foil. Mice were introduced singly into the jar, at intervals of20 or more seconds, and previously prepared volumes of 0.05ml. of challenge virus suspension were applied to the nares when the animals had reached the correct degree of anaesthesia. On a practical basis this was five seconds after the mice became completely unconscious. Approximately 120 mice were inoculated in any one hour.

Assessment of Outcome

All mice which subsequently died, up to the 7th day after inoculation, were autopsied and specific deaths from influenza virus infection were recorded. The criterion for specific influenzal death was the presence of at least 75% lung consolidation, indicated by plum coloured areas which macroscopically resembled normal liver. All mice which survived for seven days were sacrificed by exposure to excess chloroform, and the extent of lung consolidation was recorded. For concurrent experiments which were not described in this thesis, it was sometimes convenient to autopsy the mice by removal of the

ventral chest wall, but in most cases the post-mortem examination followed the dissection of the dorsal part of the chest wall.

For semi-quantal assay, specific deaths and degrees of pulmonary consolidation were allotted numerical values which were as follows : -

Death on the 3rd or 4th post-inoculation day	5.0
Death on the 5th or 6th post-inoculation day	4.5
Death on the 7th post-inoculation day	4.0

Consolidation at time of sacrifice

75 - 100%	3.0-4.0
50 - 75%	2.0-3.0
25 - 50%	1.0-2.0
0 - 25%	0.0-1.0

Between the ranges shown it was possible to recognize approximately 5% differences in extent of lung lesions. No mouse died of specific influenza virus infection before the third day after inoculation.

The numerical values were designated Mouse Scores, which title was abbreviated to M.S. for convenience.

Methods of titration of serum antibody

Neutralization Tests

Neutralization tests were carried out according to the allantois-on-shell procedure of Fazekas de St. Groth et al (1958). Non-inactivated sera were diluted in standard medium in 3 x 1/2" glass tubes distributed in racks which were then placed in an ice-

water bath at 0°C. Dilutions for final titrations were invariably 3.16 - fold in 1.08ml. volumes of medium, although in some cases preliminary titrations were carried out in 10-fold dilutions in 0.9ml. volumes. Stock challenge viruses were diluted in standard medium so that 10^3 to 10^4 ID_{50} were contained in 0.05 ml which was then added to each tube of chilled serum dilution. The mixtures of virus and serum were held in the ice-water bath for 30 minutes, and then 0.05 ml. of each mixture was added to eight replicate depressions in a plastic tray which contained a 6 $mm.^2$ piece of allantois-on-shell in 0.35 ml. of standard medium. The viral infectivity was established by controls which were carried out at the beginning and end of the experiment. The plastic trays were incubated for 72 hours at 35 - 36°C, with constant shaking. The pieces of allantois-on-shell were then removed, and 0.25 ml. of 5% fowl erythrocytes added and the trays shaken briefly. After standing for 45 minutes the presence or otherwise of virus in each of the depressions was demonstrated by the appearance or absence of haemagglutination. End-points were calculated by a modification of the method of Reed and Muench (Fazekas de St. Groth, 1955), and since all comparative tests were performed at the same time with the same dose of virus, the results of the titrations given in the text of each experiment are merely these end-points.

Anti-haemagglutinin Tests

These tests also followed the procedure of Fazekas de St. Groth et al (1958) using plastic trays containing 0.25 ml. of calcium

magnesium saline in each depression for dilution of the sera. Sera were primarily diluted to 1/10 in calcium saline and 100 units of Receptor Destroying Enzyme added and incubated at 37°C. The following pH 9.0 buffer (Aronsson and Grönwall, 1957) was then added in one-tenth volume and the serum was heated at 62.5°C for 20 minutes: -

Tris - (tris-hydroxymethyl aminomethane)	80.0 g.
H_3BO_3	8.0 g.
Disodium ethylene-diamino-tetraacetate	6.0 g.
NaN_3	0.8 g.
De-ionised and distilled water to make	1000.0 ml.

After removal of non-specific inhibitors, the sera were suitably diluted in the plastic trays, using a Takátsy loop, and 4.0 haemagglutinating doses of the homologous virus were added to each depression in a volume of 0.025 ml. The trays were shaken, and the virus and serum allowed to react for 30 minutes. Indicator fowl erythrocytes were added as 0.025 ml. of a 5% suspension, and as reported in Chapter V these had been taken from birds which were proven to be insensitive to Francis inhibitor in a 1/50 dilution of rabbit or mouse serum which had been boiled for 10 - 15 minutes.

After brief shaking, the trays were allowed to stand for 35 minutes and the pattern of settled cells was read to judge the degree of haemagglutination inhibition.

Complement Fixation Tests

The overnight procedure of Fazekas de St. Groth et al.

(1958) was used to detect the levels of complement fixing antibody in sera which had previously been inactivated at 62.5°C in a water bath, for 20 minutes. Calcium magnesium saline was dispensed in 0.25 ml. amounts in the depressions of plastic trays, and dilutions of sera were prepared using a Takátsy loop. A standard drop (0.025 ml.) containing eight units of antigen and $3HD_{50}$ of complement was added to each dilution, and the trays were held at 4°C for 18 hours. A standard drop (0.025 ml.) containing 4% sensitized sheep cells was added, and the trays were gently shaken for two hours using a mechanical shaker in a 36°C hot room. After the cells had been allowed to settle for a further two hours, the degree of haemolysis was read using the pattern of the unlysed cells, and 50% haemolysis was taken as the end-point.

Assay of Vaccines

Enzyme activity tests, and haemagglutinin pattern and photometric tests have been described in Chapter II . Gel precipitin tests and antibody absorption tests have been described in Chapter VI.

Complement Fixation Antigenicity

The overnight fixation method of Fazekas de St. Groth et al (1958) which is described above, was used to measure the specific viral complement fixing antigenicity of the vaccines. Dilutions of antigen were prepared in 0.25 ml. volumes of calcium magnesium saline in the depressions of the plastic trays, using a

Takátsy loop. In this case, a standard drop (0.025 ml.) containing 4 units of strain specific hyperimmune rabbit antiserum and $3HD_{50}$ of complement was added to each dilution. The method then followed the procedure used for the estimation of complement fixing antibody, and again the end-point chosen was 50% haemolysis.

APPENDIX 2

This appendix gives the individual results and details of most of the statistical analyses of the experiments reported in Chapters II to VI. The following abbreviations, which accord with common statistical practice, will be used throughout this Appendix : -

d.f.	Degree of Freedom
S.S.	Sums of Squares
M.S.	Mean Square
V.R.	Variance Ratio
Sig.	Significance
SSR	Residual Sums of Squares
SP	Sums of Products
SPR	Residual Sums of Products
x	Has been used for body-weight in all analyses of covariance.
y	Has been used for the particular source of variation being investigated.

TABLE A

Individual M.S. of female C57Bl and W.E.H. mice challenged with stock A/Swine virus

Age of mice	C57Bl						W.E.H.					
	Challenge Dose						Challenge Dose					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
16 weeks		3.0	4.0	2.0	0.8	0.0		4.0	3.0	0.8	0.2	0.5
		4.0	0.8	0.5	1.0	0.0		1.0	2 2	1.0	1.5	0.3
		4.0	1.0	0.5	0.5	0.0		3.0	3.5	0.0	0.3	0.2
		4.0	0.5	0.5	1.0	0.3		0.5	2.8	0.8	1.5	0.2
		4.0	0.3	1.0	0.8	0.5		3.0	1.8	1.5	1.5	1.D
19 weeks	4.5	4.0	3.5	1.0	0.0	0.0	4.0	3.0	2.5	0.8	1.5	0.0
	4.0	4.0	2.8	0.0	0.8	0.8	3.5	3.0	1.0	2.0	0.3	0.8
	4.0	3.0	2.0	1.0	1.0	0.0	4.5	2.0	2.0	1.0	1.0	0.0
	4.0	2.0	2.5	0.1	0.2	0.8	3.0	1.5	2.8	0.8	0.0	0.7
	4.0	1.5	1.8	0.3	2.0	0.4	4.5	2.5	2.0	1.5	1.3	2.0

TABLE B

Analyses of variance: 1) Dose, age and strain of mouse in 5 higher dilutions of both age groups;
2) Dose and strain for 19 weeks old mice.

Analysis (1)

Source	d.f.	S.S.	M.S.	V.R.	Sig.
Dose(D)	4	83.7194	20.9298	36.1295	***
Age (A)	1	0.0016	0.0016	0.0028	N.S.
Strain(s)	1	0.0256	0.0256	0.0442	N.S.
D-A	4	1.6114	0.4027	0.6953	N.S.
D-S	4	6.9574	1.7393	3.0024	**
A-S	1	0.0036	0.0036	0.0062	N.S.
D-A-S	4	6.1694	1.5423	2.6624	N.S.
Residual	80	46.3440	0.5793		
Totals	99	144.8324			

Analysis (2)

Source	d.f.	S.S.	M.S.	V.R.	Sig.
Dose(D)	5	92.2728	18.4546	40.2587	***
Strain(S)	1	0.0041	0.0041	0.0089	N.S.
D-S	5	2.8449	0.5690	1.2212	N.S.
Residual	48	22.0040	0.4584		
Totals	59	117.1258			

TABLE C

Weight class and M.S. of individual mice challenged with A/Swine virus. First figure in each row is weight class of mouse

Dilution of challenge virus	Male				Female			
	Age in weeks				Age in weeks			
	19	16	13	10	19	16	13	10
10^{-3}	4 1.0	3 1.5	3 3.5	4 3.0	3 3.0	2 4.0	1 3.0	1 3.5
	4 2.5	5 3.5	3 4.0	3 3.0	2 3.0	3 1.0	1 4.0	1 3.0
	4 4.5	5 0.8	3 1.5	2 2.8	3 2.0	3 3.0	3 4.0	2 3.0
	5 1.0	4 3.0	4 0.8	2 1.0	3 1.5	2 0.5	3 3.2	2 2.5
	5 2.0	5 1.5	4 3.5	3 2.5	2 2.5	3 3.0	1 1.0	1 2.0
10^{-4}	5 1.5	3 1.0	5 0.5	5 1.0	2 2.5	2 3.0	2 2.5	3 2.5
	5 3.0	4 1.0	4 1.0	1 1.0	2 1.0	1 2.2	2 2.7	4 3.8
	4 2.0	3 1.3	1 2.0	3 2.8	2 2.0	3 3.5	1 1.8	1 1.5
	5 0.3	4 1.4	3 2.2	3 1.0	3 2.8	2 2.8	2 1.3	2 2.5
	5 0.0	3 2.2	2 2.5	1 2.8	2 2.0	3 1.8	3 2.0	2 4.0
10^{-5}	5 2.0	5 4.0	5 2.5	2 3.5	2 0.8	2 0.8	2 0.8	2 0.5
	5 0.3	4 0.3	4 2.0	2 1.5	3 2.0	3 1.0	3 0.0	1 2.0
	5 0.5	3 0.5	1 1.0	1 0.2	2 1.0	3 0.0	2 1.0	2 1.0
	5 2.0	4 0.0	2 0.8	3 0.0	3 0.8	1 0.8	2 1.5	2 0.3
	3 0.1	5 1.5	3 0.0	1 0.8	2 1.5	2 1.5	2 0.0	1 1.0
10^{-6}	4 0.5	4 2.2	5 0.2	4 0.8	3 1.5	2 0.2	1 0.3	1 0.0
	5 1.0	5 0.0	4 1.2	3 0.0	3 0.3	2 1.5	2 0.5	1 1.0
	4 1.0	3 0.0	2 0.5	2 0.8	4 1.0	2 0.3	3 0.8	1 0.3
	5 0.3	4 0.8	2 0.3	3 0.4	4 0.0	5 1.5	3 1.0	1 1.0
	3 1.5	4 1.5	3 0.6	4 0.8	2 1.3	1 1.5	3 1.0	3 1.0
10^{-7}	5 0.8	4 0.0	3 0.8	2 0.3	2 0.0	2 0.5	3 0.1	2 0.1
	4 0.3	4 0.1	3 0.0	3 0.8	3 0.8	2 0.3	2 0.6	1 0.3
	3 0.1	5 0.8	3 0.0	3 0.5	3 0.0	3 0.2	2 0.2	1 0.3
	4 0.0	3 0.6	4 0.1	3 0.0	4 0.7	2 0.2	3 0.0	3 0.8
	5 1.5	3 0.0	4 0.2	3 0.3	2 2.0	3 1.0	1 0.3	2 0.0

virus in a factorial experiment.

SSy	SSy + SSRy = (B)-(C)	Estimated Variance	Variance Ratio	Signif-icance
2.3545	2.8250	2.8250	3.9577	*
0.2654	0.6831	0.2277	0.3190	N.S.
122.8050	123.1099	31.0366	43.4800	***
0.3421	0.4640	0.1547	0.2167	N.S.
4.1023	4.2278	0.3523	0.490	N.S.
7.1398	7.1434	1.7858	2.502	*
4.1977	4.1854	0.3488	0.489	N.S.
114.1120	113.4870 (C)	0.7138		
255.3188				

Analysis for weight.

Weight	1	0.6250	0.6250	0.8756	N.S.
Residual	159	113.4870	0.7138		
		114.1120			

TABLE D

Analysis of Covariance, Weight and M.S. after challenge with A/Swine

Source	d.f.	SSx	SSx + SSRx	SPxy	SPxy + SPRxy = (A)	$(A)^2$	$\frac{(A)^2}{SSx+SSRx} = (B)$
Sex(S)	1	91.1250	216.7250	-14.6475	5.7875	33.49516	0.1545
Age(A)	3	52.6150	178.2150	- 2.7815	6.0785	36.94816	0.2073
Dose(D)	4	1.5300	127.1300	- 2.2850	6.5750	43.23063	0.3401
A - S	3	6.5350	132.1350	- 0.7065	8.1535	66.47956	0.5031
A - D	12	5.7100	131.3100	- 0.7610	8.0990	65.5938	0.4995
S - D	4	1.1500	126.7500	+ 0.0150	8.8750	78.7656	0.6214
S-A-D	12	10.0900	135.6900	+ 0.4390	9.2990	86.4714	0.6373
Residual	160	125.6000		+ 8.8600		78.4996	0.6250
Total	199	294.3550		-11.8675			

N.S. Not significant

Levels of significance are denoted according to usual practice : -
*5% level, ** 1% level, and *** 0.1% level, using the Tables of Fisher and Yates (1948).

TABLE E

Individual M.S. of mice held in 2 different environments and challenged with B/Lee and A/Swine viruses.

Strain	Dose of challenge virus =	Environmental Temperature 22 - 23°C 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	30°C 10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
B/Lee		2.0	1.5	0.1	0.3	0.5	1.5	0.5	0.1	0.0	0.0
		3.0	1.0	0.5	0.1	0.1	1.0	0.2	0.5	0.0	0.0
		1.5	1.5	0.8	0.3	0.5	0.8	0.5	0.3	0.0	0.0
		1.5	1.0	0.1	1.5	0.0	0.5	0.3	0.0	0.1	0.3
		2.0	1.8	1.0	0.3	0.1	1.0	0.5	0.3	0.0	0.1
		3.0	2.5	0.1	2.0	0.3	0.1	0.5	0.5	0.0	0.0
		1.5	2.0	2.5	0.8	0.0	4.5	0.5	0.3	0.0	0.0
		2.5	2.5	0.8	1.5	0.5	1.0	0.3	0.0	0.0	0.0
		2.5	1.5	0.5	0.1	0.0	2.5	0.5	0.1	0.1	0.0
		1.5	2.5	0.3	0.0	0.0	1.5	0.8	0.3	0.1	0.0
A/Swine		5.0	4.5	3.0	2.0	0.3	4.0	1.8	2.5	1.0	0.0
		5.0	2.5	2.8	2.0	0.3	5.0	0.8	2.5	1.5	0.3
		5.0	3.5	2.5	1.5	1.3	5.0	4.5	2.5	0.5	0.3
		0.5	3.8	2.5	2.5	1.0	5.0	4.5	2.5	2.0	0.0
		4.0	3.3	2.0	1.0	2.0	2.0	4.5	2.0	2.5	0.3
		5.0	1.5	2.5	1.0	1.5	4.5	1.5	2.5	1.0	1.0
		5.0	3.0	2.0	1.5	0.0	4.0	3.0	4.0	0.1	0.0
		5.0	5.0	2.5	1.0	1.0	4.0	4.5	3.0	1.5	0.5
		5.0	4.5	4.0	2.0	2.0	5.0	2.0	1.5	1.3	2.0
		5.0	4.5	2.0	2.5	1.5	5.0	1.0	0.3	0.0	1.0

TABLE F

EFFECT OF TEMPERATURE

Analysis of variance of data from experiments with A/Swine and B/Lee in mice held at 22 - 23°C. and 30°C. (40% and 35% Relative Humidities)

Source	d.f.	SS	MS	V.R.	SIGNIFICANCE
Strain(S)	1	143.6513	143.6513	219.4825	***
Dose (D)	4	173.2541	43.3135	66.1780	***
Temp. (T)	1	15.0701	15.0701	23.0254	***
S - D	4	25.2649	6.3161	9.6502	***
T - D	4	3.7471	0.9368	1.4313	N.S.
S - T	1	0.4704	0.4704	0.7187	N.S.
S-D-T	4	1.4759	0.3689	0.5636	N.S.
Residual		118.4650	0.6545		
Totals	199	481.3988			

For interpretation of significance levels see Table D.

TABLE G

Neutralizing antibody responses expressed in $\log_{10}$ units of W.E.H. and C57Bl. mice to B/Lee vaccine at 17 and 29 days post-inoculation

Strain of mouse		Dose of Vaccine - $\log_{10}$ Haemagglutinin units				
		1.0	1.5	2.0	2.5	3.0
C57Bl	17 - day	2.0	2.2	2.4	2.9	3.0
		2.1	2.3	2.6	3.0	3.0
		2.1	2.3	2.8	3.0	3.0
		2.3	2.5	2.8	3.0	3.1
		2.4	2.5	2.8	3.0	3.3
	29 - day	2.0	2.2	2.2	2.9	3.1
		2.0	2.4	2.4	2.9	3.1
		2.4	2.4	2.5	3.1	3.4
		2.4	2.5	2.5	3.2	3.4
		2.4	2.6	2.8	3.3	3.4
W.E.H.	17 - day	2.0	2.0	2.3	2.8	2.9
		2.0	2.4	2.3	2.9	2.9
		2.3	2.4	2.4	3.1	3.4
		2.3	2.4	2.5	3.3	3.4
		2.5	2.4	2.8	3.3	3.4
	29 - day	2.0	2.2	2.0	2.9	3.0
		2.4	2.4	2.4	3.0	3.0
		2.4	2.5	2.5	3.1	3.1
		2.5	2.6	2.5	3.3	3.5
		2.6	2.6	2.8	3.4	3.5

Analysis of Variance

B/Lee vaccine - 5 doses

Source	d.f.	S.S	M.S.	V.R.	
Strain (S)	1	0.0490	0.0490	0.0591	N.S.
Time (T)	1	0.8410	0.8410	1.0144	N.S.
Dose (D)	4	141.8300	35.4575	42.7663	***
S-T	1	0.0490	0.0490	0.0591	N.S.
S-D	4	1.5660	0.3915	0.4722	N.S.
T-D	4	1.7940	0.4485	0.5409	N.S.
Residual	84	69.6460	0.8291		
Totals	99	215.7750			

TABLE H

Response of C57Bl and W.E.H. mice to A/Swine vaccine

Neutralizing antibody reciprocal titres of individual mice, expressed as logarithms to base 10.

Strain of mouse	Day 17 Vaccine doses log.10 Haemagglutinin					Day 29 Vaccine doses log.10 Haemagglutinin				
	0.0	0.5	1.0	1.5	2.0	0.0	0.5	1.0	1.5	2.0
C57Bl	1.00	1.20	1.24	1.34	1.55	1.00	1.16	1.89	1.81	2.24
	1.00	1.24	1.30	1.60	2.05	1.00	1.32	1.89	1.97	2.31
	1.00	1.30	1.46	1.66	2.08	1.00	1.56	2.06	2.04	2.31
	1.00	1.39	1.53	1.69	2.35	1.04	1.58	2.08	2.10	2.40
	1.00	1.43	1.74	1.76	2.38	1.13	1.74	2.15	2.20	2.45
	1.00	1.45	1.77	1.77	2.41	1.22	1.79	2.15	2.24	2.45
	1.07	1.49	1.79	1.99	2.42	1.67	1.85	2.18	2.39	2.48
	1.22	1.53	1.85	2.02	2.48	1.88	2.06	2.19	2.39	2.49
	1.27	1.79	2.06	2.09	2.57	1.97	2.12	2.39	2.41	2.55
	1.59	1.90	2.06	2.36	2.59	2.03	2.15	2.42	2.45	2.66
W.E.H.	1.00	1.00	1.34	1.46	1.78	1.00	1.04	1.92	1.76	2.44
	1.00	1.09	1.43	1.76	2.24	1.09	1.19	1.94	2.09	2.44
	1.00	1.13	1.49	1.79	2.24	1.34	1.60	1.94	2.12	2.45
	1.00	1.13	1.49	1.85	2.30	1.34	1.65	2.02	2.15	2.46
	1.00	1.16	1.55	1.97	2.30	1.46	1.65	2.03	2.20	2.52
	1.00	1.28	1.55	1.99	2.39	1.48	1.73	2.06	2.20	2.55
	1.16	1.43	1.73	2.06	2.40	1.49	1.76	2.06	2.20	2.55
	1.24	1.49	2.09	2.12	2.40	1.52	1.79	2.12	2.27	2.59
	1.44	1.85	2.22	2.33	2.45	1.76	1.97	2.15	2.29	2.75
	1.59	1.94	2.42	2.34	2.54	1.89	2.12	2.49	2.69	2.76

TABLE I

Analysis of Variance of the Data shown in Table H

Source	d.f.	S.S.	M.S.	V.R.	
Strain (S)	1	0.0049	0.0049	0.069	N.S.
Time (T)	1	4.3542	4.3542	61.3268	***
Dose (D)	4	30.3024	7.5756	106.6986	***
S - T	1	0.0078	0.0078	0.1099	N.S.
T - D	4	0.2107	0.0527	0.7423	N.S.
S - D	4	0.2254	0.0563	0.7930	N.S.
S-T-D	4	0.1043	0.0261	0.3676	N.S.
Residual	180	12.7711	0.0710		
Total	199	47.9808			

Analysis of variance

Source	d.f.	SS.	M.S.	V.R.	Signif-icance
Dose(D)	1	3.3702	3.3702	5.1399	*
Sex (S)	1	0.0708	0.0708	0.1080	N.S.
D-S	1	0.0040	0.0040	0.0610	N.S.
Residual	76	4.9835	0.6557		
	79	8.4285			

TABLE J

Anti-haemagglutinin responses of male and female C57 Bl mice

$\log_{10}$ reciprocal titres of sera from individual animals injected with A2/Singapore/1/57M vaccine.

Sex	Male		Female	
Dose of Vaccine	400 Haemagglutinin Units	800 Haemagglutinin Units	400 Haemagglutinin Units	800 Haem-agglutinin Units
Titres	1.96	1.60	2.22	1.55
	2.08	2.02	2.30	1.78
	2.08	2.20	2.15	1.81
	2.20	1.99	2.45	2.05
	1.96	2.02	2.52	1.77
	1.60	1.60	2.07	2.00
	1.84	1.93	2.00	2.15
	1.69	1.30	2.56	2.56
	1.96	2.02	2.49	2.22
	1.60	1.90	2.30	2.22
	2.05	1.84	2.38	2.64
	1.93	2.04	2.15	2.71
	1.90	1.90	2.22	2.53
	1.66	2.02	2.30	2.38
	1.90	2.02	2.07	2.19
	1.90	1.30	2.19	1.63
	1.81	1.30	2.38	2.52
	1.84	1.69	2.45	2.38
	1.45	1.75	2.15	2.38
	1.60	1.66	2.15	2.56

TABLE K

Weight class and M.S. of individual immunized mice challenged with A/Bel virus. First figure in each row is weight class of mouse.

Dose of Haccine. Haemagglutinin Units	Male Age in weeks						Female Age in weeks					
	12		9		6		12		9		6	
2048	4	0.0	5	0.0	2	0.0	3	0.0	4	0.0	2	0.3
	4	0.0	4	0.6	3	0.0	4	0.0	3	0.0	2	0.3
	3	0.0	3	0.1	3	0.0	3	0.0	3	0.0	1	0.7
	4	0.0	5	0.0	3	0.0	4	0.0	3	0.0	1	3.0
	4	0.2	4	0.0	3	0.0	4	0.0	3	0.0	1	0.0
	5	0.0	3	0.5	2	0.0	3	0.0	4	0.0	2	0.0
1024	5	0.2	5	0.8	2	0.0	4	0.0	3	0.0	1	0.0
	5	0.5	5	0.0	2	0.3	3	0.0	3	0.0	1	0.6
	3	0.0	5	0.0	2	0.0	4	0.0	2	0.0	1	0.8
	3	0.0	5	0.0	3	0.1	4	0.0	3	0.0	1	0.0
	5	0.5	3	0.0	2	0.3	4	0.0	3	0.0	1	0.0
	4	0.0	3	0.0	2	0.7	2	0.3	5	0.0	1	0.8
512	5	0.0	5	1.2	2	0.0	3	0.0	2	0.0	1	0.0
	5	0.0	5	0.0	3	0.0	4	0.3	1	0.0	1	2.0
	5	0.1	5	1.1	5	0.3	4	0.4	5	0.0	2	0.5
	5	0.7	5	0.0	3	0.2	5	0.0	2	0.0	1	0.0
	5	1.0	5	0.0	2	3.2	4	0.0	2	0.6	1	0.8
	5	1.0	4	0.2	3	0.0	3	0.0	2	0.0	1	1.2
256	4	0.0	5	3.0	3	4.0	3	1.4	3	4.0	2	1.4
	4	0.0	5	2.4	1	0.2	3	0.2	3	0.5	1	3.1
	3	3.7	3	0.2	2	0.2	4	0.2	3	0.8	1	0.5
	3	4.0	5	0.0	2	1.0	4	1.4	4	0.0	2	0.3
	4	0.8	5	2.2	3	0.0	4	0.0	4	1.0	1	0.7
	5	0.8	5	0.0	4	0.5	3	0.0	2	1.5	1	2.6
128	3	2.0	4	3.0	3	1.8	3	0.9	4	0.4	1	1.0
	4	1.6	4	3.2	1	4.0	4	0.7	2	4.0	1	2.3
	4	0.0	4	0.8	2	2.0	4	0.5	2	4.0	1	0.0
	4	2.0	5	0.5	1	2.4	4	0.0	3	1.4	1	0.8
	5	0.8	5	0.0	1	3.2	4	0.0	3	0.0	1	0.0
	3	0.8	5	0.0	2	0.1	3	3.1	3	1.2	1	0.8
64	4	1.4	4	1.1	3	0.5	2	1.0	2	4.0	1	0.8
	5	0.0	5	2.0	3	1.3	3	0.5	2	0.3	1	2.0
	4	1.4	4	4.0	3	1.0	3	0.5	3	3.5	1	0.8
	4	1.0	5	0.3	1	2.5	2	4.0	3	0.6	1	0.5
	5	0.0	5	1.8	5	1.0	4	2.0	3	0.8	1	0.5
	3	2.3	4	2.4	3	1.4	4	0.5	3	0.0	1	2.6
32	3	0.7	3	4.0	4	4.0	2	3.0	2	4.0	1	2.8
	5	0.1	5	0.0	3	2.8	2	1.6	3	4.0	2	3.7
	3	0.0	4	1.5	2	0.7	2	1.7	5	4.0	2	0.0
	4	0.6	3	2.7	3	1.5	3	0.5	2	4.0	1	4.0
	4	4.0	3	0.7	2	3.2	4	2.3	3	0.3	1	1.5
	5	0.3	4	0.3	2	2.4	3	0.8	1	0.4	2	2.2
16	5	0.4	4	0.5	2	3.2	2	3.8	2	3.4	1	2.2
	3	0.3	4	0.8	3	1.5	3	1.2	2	1.0	1	3.5
	3	4.0	5	0.7	2	2.2	3	1.0	2	4.0	1	3.5
	4	4.0	3	4.0	2	3.5	2	1.8	2	4.0	1	3.0
	4	0.3	4	4.0	2	0.6	1	2.1	3	4.0	1	0.8
	4	4.0	4	0.0	3	0.3	2	4.0	3	4.0	1	0.8

TABLE I

Analysis of covariance: Effect of Age and Sex of mice on challenge with A/Bel virus in immunized mice. Body-weight class as covariate.

Source	d.f.	S.S.x	S.S.x + S.S.R.x=(A)	S.P.xy	S.P.xy+ S.P.R.xy=(B)	$(B)^2$
Sex(S)	1	108.7812	238.6145	- 8.5427	-55.4760	3077.58658
Age(A)	2	201.2153	331.0486	- 21.6441	-68.5774	4702.85979
Dose(D)	7	13.1354	142.9687	- 37.3726	-84.3059	7107.48477
A - S	2	4.9375	134.7708	- 2.8261	-49.7594	2475.99789
A - D	14	13.5625	143.3958	+ 1.6330	-45.3003	2052.11718
S -D	7	10.1910	140.0243	- 2.0351	-48.9684	2397.90420
S-A-D	14	6.1737	136.0070	- 2.6128	-49.5461	2454.81603
Residual	240	129.8333		- 46.9333		2202.73465
Totals	287	487.8299		-120.3337		

Analysis for Weight

Source	d.f.	S.S.	M.S.	V.R.	Sig.
Weight	1	16.9659	16.9659	14.3559	***
Residual	239	282.4524	1.1818		
Totals	240	299.4183			

L

$\frac{(B)^2}{A} - (C)$	S.S.y	S.S.y+S.S.R.y - C - D	Estimated Variance	V.R.	Significance
12.8977	0.6709	4.7391	4.7391	4.01	*
14.2059	6.4293	9.1893	4.5947	3.888	*
49.7136	165.2908	132.5431	18.9347	16.029	***
18.3719	9.0252	7.6192	3.8096	3.2224	*
14.3108	10.6907	13.3458	0.9533	0.8066	N.S.
17.1249	11.0905	10.9315	1.5616	1.321	N.S.
18.0492	9.7693	8.6860	0.6204	0.505	N.S.
16.9659	299.4183	282.4524=(D)	1.1818(with 239 d.f.)		
	512.3850				

TABLE M

Weight class and M.S. of immunized mice challenged with A/Swine virus. First figure in each row is weight class of mouse.

Dose of Vaccine	Male				Female			
	Age in weeks				Age in weeks			
	15	12	9	6	15	12	9	6
3000	5 0.0	4 0.0	1 0.3	1 0.0	1 0.0	1 0.0	1 0.0	1 0.0
	5 0.0	3 0.0	3 0.0	1 0.3	2 0.0	1 0.0	1 1.3	1 0.0
	3 0.0	4 0.0	1 0.0	1 0.0	2 0.0	2 0.0	2 0.0	1 0.0
	3 0.0	2 0.0	2 0.0	1 0.0	3 0.0	1 0.0	1 0.0	1 0.0
	5 0.0	3 0.0	1 0.0	1 0.3	2 0.0	1 0.0	2 0.0	1 0.3
300	4 0.0	5 0.0	3 0.0	1 0.0	3 0.0	2 0.0	1 0.0	1 0.0
	4 0.1	2 0.0	2 0.0	1 0.0	1 0.5	1 0.0	2 0.1	1 0.1
	4 0.1	4 0.3	1 0.5	1 0.7	2 0.0	1 0.0	2 0.0	1 0.0
	4 0.0	4 0.3	3 0.5	1 0.0	3 0.0	1 3.0	1 0.0	1 0.3
	5 0.0	1 1.0	2 0.3	1 0.3	2 0.0	2 0.4	1 0.1	1 0.3
30	5 0.0	2 0.5	2 0.3	1 4.0	3 0.5	2 0.0	1 0.3	1 2.0
	4 0.5	4 1.0	4 1.0	1 0.7	2 2.0	1 1.0	2 1.0	1 4.0
	4 0.0	3 2.3	1 1.5	1 1.8	3 1.0	2 1.8	1 0,8	1 1.5
	4 0.8	4 0.5	1 1.0	1 0.8	1 0.5	1 0.3	1 2.0	1 0.3
	3 1.0	2 0.3	1 3.0	1 1.6	3 0.3	2 1.0	1 3.0	1 2.0
3	5 1.8	3 3.0	3 1.0	1 4.0	2 3.0	2 3.0	2 3.5	1 4.0
	5 0.7	5 0.8	3 1.0	1 2.8	2 2.0	1 1.0	2 3.0	1 1.0
	3 1.5	2 1.5	2 1.5	1 2.2	2 3.8	1 1.3	2 4.0	1 4.0
	4 2.0	4 1.0	1 4.0	1 2.8	4 3.0	2 0.1	1 3.2	1 4.5
	3 3.0	1 2.8	2 1.5	1 4.5	2 0.5	1 3.0	2 3.8	1 0.5
0.3	5 0.3	3 1.5	1 3.0	2 3.3	3 3.0	3 3.3	1 2.0	1 4.0
	2 4.0	3 2.0	1 2.3	1 3.5	1 4.0	3 4.5	1 4.0	1 1.0
	4 0.5	5 2.5	4 0.2	1 1.0	2 3.0	1 3.0	1 2.5	1 3.5
	5 1.7	3 0.8	2 1.5	1 3.0	1 3.5	1 4.0	2 2.0	1 4.0
	4 2.0	3 1.8	2 3.0	1 3.8	4 2.0	1 4.0	1 3.0	1 2.0
0.03	4 3.0	3 2.0	1 1.0	1 2.0	3 2.5	2 2.5	1 1.8	1 3.0
	4 3.0	2 1.8	2 3.5	1 1.4	2 3.5	1 4.0	2 3.3	1 1.5
	5 1.0	4 0.5	3 2.3	1 3.5	2 0.7	2 3.0	2 4.5	1 3.5
	4 2.5	4 2.0	2 3.5	1 2.5	2 3.8	1 4.0	1 3.0	1 3.0
	3 2.0	2 3.8	1 2.6	1 2.5	2 4.5	3 2.0	2 3.5	1 1.5

TABLE N

Analysis of covariance: Effect of Age and Sex of mice on challenge with A/Swine virus in immunized mice. Body-weight class as covariate.

Source	d.f.	S.S.x	S.S.x + S.S.Rx=(A)	S.P.xy	S.P.xy + S.P.R.xy=(B)	$(B)^2$
Sex (S)	1	59.0042	175.4042	-22.9075	-56.5675	3199.88206
Age (A)	3	149.5459	265.9459	-31.4184	-65.0784	4235.19815
Dose (D)	5	0.7209	117.1209	+ 9.0758	-24.5842	604.38289
A - S	3	33.5791	149.9791	- 8.6358	-42.2958	1788.93470
A - D	15	2.5291	118.9291	+ 1.4609	-32.1991	1036.78204
S - D	5	1.0208	117.4208	+ 0.5350	-33.1250	1097.26562
S-A-D	15	1.6959	118.0959	+ 1.7483	-31.9117	1018.3566
Residual	192	116.4000		-33.6400		1132.9956
Total	239	364.4959		-83.8017		

Analysis for Weight

Source	d.f.	S.S.	M.S.	V.R.	Sig.
Weight	1	9.7336	9.7336	12.892	***
Residual	191	144.2144	0.7550		
Totals	192	153.9480			

$\frac{(B)^2}{A} - (C)$	SSy	S.S.y+SSRy -C -D	Estimated Variance	V.R.	Significance
18.2429	8.8935	0.3842	0.3842	0.509	N.S.
15.9250	6.8603	0.6689	0.2230	0.295	N.S.
5.1603	283.3563	287.9296	57.5859	76.2727	***
11.9278	3.7908	1.5966	0.5322	0.705	N.S.
8.7176	16.1247	17.1407	1.1427	1.514	N.S.
9.3447	7.8905	8.2794	1.6559	2.193	N.S.
8.6231	11.1352	12.2457	0.8164	1.081	N.S.
9.7336	153.9480	144.2144=(D)	0.7550(with 191 d.f.)		
	491.9993				

TABLE C

Results of challenge with three levels of A/Swine virus in mice immunized with three doses of A/Swine vaccine

Individual M.S. and body-weights (Less 30 grams). Mean weight of mice 34.4.grams.

Vaccine Dose Haemagglutinin Units	Vaccine B Mice challenged with			Vaccine C Mice challenged with		
	1000 M.S.2.5	316 M.S.2.5	100 M.S.2.5	1000 M.S.2.5	316 M.S.2.5	100 M.S.2.5
80	2.5-10	1.0 -7	0.8 -7	4.5 -8	0.8 -6	1.0 -7
	0.8- 5	2.3 -6	2.0 -6	4.5 -5	0.1 -6	2.8 -6
	3.0- 4	0.0 -4	0.0 -5	0.5 -4	0.8 -5	1.3 -5
	0.8 -3	5.0 -3	2.0 -4	5.0 -3	4.0 -4	0.8 -4
	4.5 -1	4.5 -2	1.5 -3	0.8 -2	2.5 -4	0.3 -3
	5.0 -1	1.3 -1	4.5 -2	1.3 -1	4.5 -3	3.5 -2
160	1.5 -9		0.3 -7	2.5 -8		2.8 -7
	4.5 -5		2.0 -6	1.0 -5		0.8 -6
	1.3 -4		1.5 -5	4.5 -4		2.3 -5
	4.5 -3		1.8 -4	1.0 -3		1.5 -4
	5.0 -2		1.5 -3	4.5 -2		0.3 -3
	0.8 -1		1.3 -2	1.0 -1		4.0 -2
320	1.3 -9	0.3 -7	1.5 -7	0.5 -8	0.1 -7	1.0 -6
	4.0 -5	0.1 -6	0.1 -6	5.0 -6	3.3 -6	4.0 -6
	0.3 -4	4.0 -4	0.5 -5	1.0 -4	0.3 -4	1.0 -5
	0.3 -3	0.5 -3	0.0 -4	0.1 -3	1.0 -3	0.0 -4
	4.5 -2	1.0 -2	0.3 -3	0.8 -2	2.0 -2	0.2 -3
	4.5 -1	0.1 -2	0.3 -2	5.0 -1	0.3 -1	0.0 -3

TABLE P

Analysis of Variance of data in Table O. Rows of individual M.S. and body-weight corresponding to 80 and 320 units of haemagglutinin

Source	d.f.	SSx	SSx +SSRx = (A)	SPxy	SPxy+SPRxy = (B)	$(B)^2$	$\frac{(B)^2}{A} = C$ *
Challenge(C)	2	3.8611	309.6944	- 7.9223	-42.5056	1806.72603	5.8339
Dose (D)	1	0.1250	305.9583	+ 1.3041	-33.2792	1107.5052	3.6198
Vaccine (V)	1	0.1250	305.9583	- 0.0209	-34.6042	1197.45066	3.9138
C - D	2	0.5834	306.4167	+ 0.7501	-33.8332	1144.68542	3.7357
C - V	2	0.5834	306.4167	+ 0.0584	-34.5249	1191.96872	3.8900
D - V	1	0.3472	306.1805	- 0.3124	-34.8957	1217.70988	3.9771
C-D-V	2	1.1944	307.0277	- 0.3918	-34.9751	1223.25762	3.9842
Residual	60	305.8333		-34.5833		1196.00464	3.9106
Totals	71	312.6528		-41.1181			

C	SSx	SSx+SSRx - C - D	Estimated Variance	V.R.	Significance
	20.8870	18.9637	9.4819	3.3338	*
	13.6068	13.8976	13.8976	4.8863	*
	0.0035	0.0003	0.0003	0.0001	N.S.
	1.3853	1.5602	0.7801	0.2743	N.S.
	0.2813	0.3019	0.1510	0.0531	N.S.
	0.5036	0.4371	0.4371	0.1537	N.S.
	1.1007	1.0271	0.5136	0.1806	N.S.
	174.5617	170.6511 (D)	2.8442(with 59 d.f.)		
	212.3299				

Analysis for Weight

Source	d.f.	S.S.	M.S.	V.R.	Sig.
Weight	1	3.9106	3.9106	1.352	N.S.
Residual	59	170.6511	2.8924		
Totals	60	174.5617			

TABLE Q

Individual data from mice immunized with B/Lee vaccines and challenged with 100 M.S. 2.5

Level of Haemagglutinin	Vaccines. Formaldehyde	Ultra-violet irradiation	Heat
400	2.0	0.8	3.0
	2.0	3.0	2.5
	0.5	4.0	4.0
	3.0	2.5	2.0
	2.0	4.0	2.5
	1.0	2.5	4.0
	0.3	3.5	0.5
	0.3	1.3	0.5
	0.3	0.8	1.0
	0.1	3.0	2.0
200	4.5	3.5	2.0
	4.0	4.5	4.0
	1.5	4.5	2.5
	0.5	4.5	3.5
	0.3	4.0	4.0
	0.8	4.0	3.0
	0.3	2.5	0.3
	2.5	3.3	1.0
	0.3	2.0	3.0
	1.5	2.0	2.3
100	4.5	3.5	4.0
	3.3	4.5	3.5
	0.3	4.5	5.0
	3.0	4.5	1.5
	0.1	3.0	2.5
	3.0	4.0	1.5
	3.0	2.5	2.0
	2.5	3.3	4.0
	3.5	3.0	3.5
	1.0	3.3	2.5

TABLE R

Analyses of variance of results with Vaccines B and C and Vaccines B and D.

Vaccines B and D

Source	d.f.	S.S.	M.S.	Variance Ratio	Significance
Vaccine(V)	1	11.0081	11.0081	6.767	*
Dose (D)	2	10.8523	5.4261	3.356	*
V - D	2	0.6044	0.3022	0.186	N.S.
Residual	54	87.8410	1.6267		
Totals	59	110.3058			

Vaccines B and C

Source	d.f.	S.S.	M.S.	Variance Ratio	Significance
Vaccine(V)	1	32.8550	32.8550	22.9418	***
Dose (D)	2	13.8810	6.9405	4.8464	*
V - D	2	1.1940	0.5970	0.4169	N.S.
Residual	54	77.3360	1.4321		
Totals	59	125.2660			

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